ASSAY AND PURIFICATION OF COXSACKIEVIRUS B3

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An abstract of a thesis by
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The problem. Which of the available methodologies for the purification for Coxsackie virus B3 (CB3), a Picornavirus provide the highest degree of purity with the greatest yield.

The procedure. Quantitative methods for the assay and purification of CB3 were developed. CB3 was propagated in buffalo green monkey (BGM) cells in tissue culture. The virus was released from the infected BGM cells by various methods. Purification methods included precipitations, differential, isopycnic, and rate zonal centrifugation. Virus purity was assessed by SDS-polyacrylamide gel electrophoresis.

Findings. The plaque assay for CB3 was found to be reproducible and quantitative. The dose response curve of the assay was linear between 5 and 800 plaques on a 55 cm^2 tissue culture dish. Maximum yields of CB3 were obtained from infected cells by treatment with sodium dodecyl sulfate (SDS). The overall yield of CB3 purified by isopycnic banding in cesium chloride was approximately 40% of the virus in the initial cell lysate. As judged by polyacrylamide gel electrophoresis in SDS, the purified virus, following rate zonal centrifugation in sucrose, was greater than 90% homogeneous.

Conclusions. The virus yields and purity were suitable for the study of virus-cell adsorption interactions.

Recommendations. Further studies should be done using these techniques in an attempt to increase the amount of protein and virus applied to isopycnic and rate zonal gradients.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Of the nineteen families of viruses with members affecting humans, the family Picornaviridae (Pico=small; RNA=ribonucleic acid) contains the smallest (27-30 nm diameter) of all known RNA viruses. Picornaviruses are nonenveloped RNA viruses which collectively form the second largest group of human viral pathogens. There are 185 picornaviruses which affect humans and many more affecting other vertebrates as well as plants. Included in the Picornaviridae are such viruses as the well known polioviruses (PV), hepatitis A virus (HAV), and the human rhinoviruses (HRV) to which, in part, the common cold is attributed. Nonhuman picornaviruses of importance include the Mengo viruses, and the Foot-and-mouth disease virus (FMDV).

Historically Significant Developments

The Germans Loeffler and Frosh (1898) were among the first investigators to work with animal viruses (1). Their subject was the causative agent of foot-and-mouth disease (FMD) which was presumed to be a bacterium. They established that the causative agent, known as a "corpuscular entity," was filterable through microporous filters known to retain even the smallest known bacteria (1). Together with many other "filterable agents," FMD was established as a disease of viral origin and the properties of the virus placed it into the family Picornaviridae (2). In 1909 Landsteiner and Popper (3) demonstrated, using spinal cord and fecal ultrafiltrates for intraperitoneal

inoculation of monkeys, that a virus caused poliomyelitis. However, it was not until 1955 that it was established that poliomyelitis was a disease caused by a picornavirus (4). The propagation of poliovirus in cultures of nonneural human and monkey kidney tissue was accomplished by Enders, Weller, and Robbins in 1945 (5). This finding led directly and rapidly to the development of the Salk and Sabin vaccines (6). Propagation of viruses in tissue culture also brought about the intense study of the molecular biology of picornaviruses. In 1954, using poliovirus, Renato Dulbecco developed the plaque assay for viral infectivity (7). This assay now extended to many other viruses is one of the most fundamentally important procedures in virology. Methods for the purification and crystallization of poliovirus particles were developed by Schaffer and Schwerdt in 1955 (8). A source of pure crystallized virus made it possible to analyze the structure of picornaviruses by X-ray crystallography (9).

During studies in which newborn mice rather than adult mice were used as hosts for poliovirus, Dalldorf et al., in 1948, isolated two new viruses (10). These viruses became the first members of a nonpoliovirus subgroup within the Picornaviridae family. These viruses were isolated from the fecal material obtained from each of two children with flaccid paralysis in Coxsackie, New York (11). The so called "coxsackieviruses" (CV) were later found to infect the enteric tract of humans. With this finding it became apparent that poliovirus was not the sole cause of viral enteric infections in man.

The discovery of the subgroup echoviruses (enteric cytopathic human orphan viruses), the third designation within the Picornaviridae family, was brought about by the introduction of better tissue culture techniques (12). The first echoviruses were isolated from human feces obtained during epidemiologic studies of poliomyelitis. The echoviruses were frequently found in normal individuals free from clinical disease subsequently these viruses were not clearly associated with any known human disease. These viruses were appropriately named "orphan" viruses since they were not associated with any known disease. Time and efforts have changed the orphan status of Echoviruses. They are known to be the causative agents of respiratory syndromes, febrile rashes, and central nervous system infections (13).

The rhinoviruses, (rhino=nose), the last of the subgroup designations of the human Picornaviridae, were discovered by Price in 1956 as a result of studies of mild upper respiratory tract infections that resembled the common cold (14). Kruse in 1914 showed that the common cold was transmissible to man by a filterable agent probably a virus (15). In 1953, Andrewes et al. reported a method for cold virus propagation (16). Further studies by Andrews and Dowling et al. showed that the common cold was caused by a large number of similar viruses (17,18,19). Since the original isolation and characterization in 1956, 115 immunologically distinct but biologically related viruses have been isolated and classified as rhinoviruses (20).

Picornavirus Structure

The morphology, physical and chemical characteristics of the picornaviruses differ only slightly from group to group in size and structure (21). The picornaviral capsid has a regular arrangement of subunits, as shown by negative staining, that are consistent with the icosahedral symmetry of the virion (21). The diameter of the picornaviral virion is 27-30 nm. The RNA genome of the virion is single stranded, of positive sense and 7.7 kilobases in size with a G+C composition of 46 moles %. The coxsackieviruses differ significantly in base composition with only 5% base sequence homology with polioviruses (21). The particle mass of the picornavirus virion is 1.1×10^{-17} grams, as determined by density and mass calculations, and has a molecular weight of $8\text{-}9 \times 10^{6}$ daltons (22). RNA makes up 30% of the virion by weight and the remainder of the mature infectious structure is composed of four major and one minor species of virus structural protein (virus proteins, VP,1-4; and VPg) (23). VP1-3 are found on the surface of the capsid. VP4 is associated internally with the inner surface of the capsid and the RNA, and VPg is covalently attached to the 5' end of the RNA (21). The RNA codes for at least three nonstructural proteins, two of which are proteases and the other an RNA transcriptase.

Picornavirus Classification

The family Picornaviridae consists of four genera, Enteroviruses, Cardioviruses, Rhinoviruses, and Apthoviruses. The Enterovirus and the Rhinovirus genera have members affecting man. Although they are

physically very similar, the picornaviruses can be divided by means of the location or the site where they normally infect and multiply within humans or other hosts (24). The enteroviruses normally inhabit the enteric tract of man and other animals. The apthoviruses infect hooved animals. The rhinoviruses infect the nasopharyngeal region of man and other animals. The cardioviruses, as the name implies, infect cardiac tissue.

The genus Enterovirus includes the polioviruses (3 serotypes), the coxsackieviruses (23 A serotypes and 6 B serotypes), the echoviruses (enteric cytopathic human orphan viruses, 32 serotypes), the human enteroviruses (serotypes 68-72), and the human hepatitis A virus. There are additionally, 37 nonhuman enteric viruses classified in this genus. Species designations are not made. Coxsackieviruses are divided into groups A and B, a division which originally was made based on the types of lesions produced in newborn and suckling mice. Group A viruses produce a diffuse myositis with acute inflammation and necrosis of fibers in voluntary muscles (25). Focal areas of degeneration in the brain, localized necrosis of skeletal muscle, inflammation of the dorsal foot pads, pancreas and occasionally the myocardium are all symptoms caused by infection of newborn mice with the group B coxsackieviruses (25).

The genus Cardiovirus contains the Colombia SK, encephalomyocarditis (EMC), Maus Eberfeld (ME) and the mengoviruses. All the viruses in this genus are of one serotype and are considered to

be strains of the EMC virus (24). These viruses do not regularly infect humans.

The genus Rhinovirus, with members infecting the nasopharyngeal region of man, has 89 recognized serotypes with a number of additional candidate viruses known. The total number of viruses in this genus is most likely 115 (20). Rhinoviruses are differentiated from all other picornavirus genera by their rapid inactivation at pH less than 7.0.

The fourth subdivision is the genus Apthovirus. This genus contains the foot-and-mouth disease virus which is itself comprised of 7 immunotypes. Fifty-three individual viruses (within these seven types) have been designated by complement fixation (24). All the immunotypes are highly acid labile and are inactivated at pH greater than 7 (24). A number of viruses including equine rhinoviruses 1 and 2, and a collection of insect and plant picornaviruses have not yet been assigned genera.

Picornavirus Multiplication

Picornaviruses replicate in living cells. The multiplication of picornaviruses has been exceptionally well-studied and is the subject of many reviews (26). Whether a particular picornavirus can attach to and multiply in a cell is known as tropism. Tropism is, for the most part, determined by the presence or absence of a cell-specific receptor for the virus. The specific cellular receptor plays an important early role in viral multiplication as it initiates the process of viral endocytosis. Endocytosis results in the delivery of the viral genome

into the cell cytoplasm where transcription, translation, and replication of the genome occurs. The virus shuts down host protein synthesis by an as yet unknown mechanism and produces viral polysomes using the virus RNA, the infected cell's ribosomes and protein synthesizing components. Translation of the picornaviral mRNA yields a large polyprotein which is cleaved to both structural and nonstructural proteins. Three nonstructural proteins of virus origin include, a protease required for cleaving viral structural proteins from a precursor, a replicase necessary for initiating RNA synthesis from the viral RNA, and an RNA polymerase (second replicase) required for the transcription of (+)RNA of the virus to the complimentary template (-)RNA. Extensive transcription of (-)RNA leads to an exponential rise in the amount of genomic (+)RNA. The entire (+)RNA coding region is translated into a polyprotein which is cleaved several times by viral proteases to produce viral structural peptides. The accumulation of structural polypeptides initiates an autocatalytic assembly of structural peptides (protomers) to form capsomers and then the entire viral capsid. Insertion of RNA occurs at a late stage of capsid assembly. Finally, a maturation cleavage of one of the capsid protomers (a cleavage of protein outside the cell) results in the infectious virus. The virions are released by infection-mediated cell lysis, presumably by the cell's own lysosomes. The time required for one complete cycle of replication depends on the specific picornavirus, the host cell, and the multiplicity of infection (MOI). The time required

for poliovirus to replicate as determined from one step growth curves is 3.0 h (24), and for CB3 7 to 8 h (Collin Teugh, unpublished data).

Soon after picornaviral infection the rate of cellular RNA synthesis begins to decrease. Shortly thereafter the rate of cell specific protein synthesis declines and stops altogether. These processes have mechanisms which are as yet not understood. Picornavirus infection adversely affects the cell morphology, macromolecular synthesis, and causes several specific cytopathic effects (CPE). Nuclear material loses its normally homogeneous microscopic form and collects along the inside of the nuclear membrane, this process, known specifically as margination of chromatin, is very apparent at 2 h post infection (27). Two to three hours post-infection, vesicles appear in the cytoplasm, near the nuclear envelope, and spread outward through the cytoplasm (28). In the late stages of infection initiated by coxsackieviruses, the cell membrane ruptures or dissolves at hundreds of sites before total dissolution of the cell (29). Cell lysis is the last of the typical cell culture CPE by any of the picornaviruses.

Cellular receptors for viruses have been of great interest because of their relationship to tropism. Recently, the human rhinovirus and the poliovirus receptor molecules have been identified (30,31). Using criteria of specific binding of a purified cellular receptor for a virus and, also, the process of transfection which can confer virus binding to a cell which normally would not bind virus, the receptor for the major group of human rhinoviruses and for poliovirus type I were identified (30,31). Both receptors are members of the

immunoglobin super family, which includes CD4 the receptor for HIV and Intracellular Adhesion Molecule, 1 (ICAM 1) (32). The receptor for rhinoviruses is the same molecule which binds to the lymphocyte integrin LFA-1. LFA-1 is a cell surface lymphocyte function-associated antigen (33). LFA-1 plays an important role in immunological inflammatory functions (33). The poliovirus receptor molecule (PRM) does not have a known cellular function.

During the studies to establish receptor groupings of viruses and to identify the receptor molecules, relatively large amounts of many purified viruses were required (34). An efficient purification procedure was required to obtain each of these viruses. Remarkably the 89 grouped rhinoviruses have only two receptor types, the major rhinovirus group, encompassing some 79 viruses, was found to bind to ICAM-1. The minor group of 10 viruses and several unrelated to human rhinoviruses were found to bind to another protein unrelated to ICAM-1.

The purification of a HeLa cell receptor for CB3 was described by Mapoles et al. (35). This receptor, R_p-a, was found in a virus receptor complex (VRC) extractable from cells using detergents. The initial studies concerned with the cellular molecules responsible for tropism of CB3 were not supportive of virus binding to cells through a member of the immune family super group. The Rp-a receptor is composed of glycoproteins which may form a pentameric cell membrane pore that is complimentary to the postulated circular canyon on the CB3 virion (36). This contrasts directly with the immune family super group receptors

which protrude from the surface of the cell membrane and contain several external domains (32).

Again, large amounts of purified CB3 were required to identify the receptor and affect its purification. The study by Mapoles which used 10^8 cells, each cell containing over 10^5 receptors, purification required initially 10^{12} to 10^{13} virus particles to saturate the cell receptor population. Since approximately 100 virions emanate from each infected cell in the system described by the authors, 10^{10} to 10^{11} cells were required to obtain sufficient virus.

A goal of the virus laboratory at the University of Osteopathic Medicine and Health Sciences has been the identification of the simian cell receptor for CB3. It was necessary to develop efficient means for host cell production, virus production and purification of the CB3 virus from simian cells. This report is concerned with the purification of CB3 produced in a simian cell line, BGM.

LITERATURE SURVEY

Differing techniques for purifying, in large amounts, picornaviruses appropriate for the study of the virus-cell interactions and virus structural polypeptides were the focus of review for the compilation of this literature survey. Most of the methods for viral production and purification have been very commonly used for members of many families of viruses. An example of a hypothetical but common method for the purification of a nonenveloped virus follows. Cells infected by a virus are frozen and thawed one or more times to release most of the virus from cellular debris. The cellular debris is pelleted by centrifugation at approximately 10,000 xg, the supernatant is saved and the pellet is resuspended in a small amount of a buffer and sonicated. This resuspended-sonicated mixture is centrifuged again (10,000 xg) and all the supernatants are combined. The virus in this fraction, which is often referred to as clarified cell lysate, is then pelleted by centrifugation (approximately 150,000 xg). The supernatant is discarded and the pellet is resuspended in a small amount of a buffer. The viral suspension is then banded by isopycnic centrifugation. Note that several techniques and medias may be employed in this centrifugation. Following location of the infectious virus, the last step of many purifications is a rate zonal centrifugation through sucrose, glycerol, or ficoll gradients.

Crude viral lysate taken through these steps have often resulted in highly purified, nearly homogeneous virus preparations. Unfortunately, not all viruses conform to this cascade of steps and often changing the cell host for the virus, the strain of the virus used, or other cultural parameters requires an entirely new procedure to be developed for the virus purification. When purity and high titer of a particular virus is needed, researchers must often adapt or develop a new procedure specific for the virus in question. The following section includes procedures and analyses of several published techniques used to obtain highly purified picornaviral preparations.

Crowell and Philipson (37) used suspension cultures of HeLa cells to grow CB3 strain Nancy and Poliovirus type 1 (PV1). The cells at near maximum density were harvested, concentrated by centrifugation, and resuspended in a low ionic strength neutral pH buffer. Virus was added to the cells at MOI's of 10 to 50 and the mixture incubated for 1 h at 37 C with stirring to affect adsorption. After an additional 8 h of incubation, the cells were harvested by centrifugation, washed once, resedimented, and resuspended in the same buffer. The virus (CB3 or PV1) was released from the HeLa cells by three cycles of freezing at -20 C and thawing. The cell debris was removed by centrifugation. The virus in the supernatant fraction was purified by overlaying the released virus, free of particulate cell debris, onto cushions of CsCl at a density of 1.40 g/ml in centrifugation tubes. Centrifugation was for 3 h at 38,000 rpm in a Beckman SW-41 swinging bucket rotor. The virus was collected in a single fraction by bottom puncture of the

centrifuge tube. The virus rich fractions from several such centrifugations were pooled, dialyzed and again centrifuged (10,000 xg for 15 min) to remove coarse particle aggregates. Solid CsCl was added to the virus fractions. The virus-CsCl suspension was centrifuged for 16 h at 38,000 rpm in the SW-41 to achieve equilibrium. The bottom of the tube was punctured and 2-3 drop fractions were collected, diluted with buffer, and assayed for infectivity. The peak fractions of virus infectivity were pooled, dialyzed and frozen in liquid nitrogen. This purified virus was then used to study its attachment to the HeLa cell membranes. The procedures reported by Crowell required titers of approximately $8x10^9$ viruses/ml, with a range of MOI's of 18 to 200 used in adsorption experiments. Total yields of virus and the number of viruses on a per cell basis were not reported. The virus preparations were over 90% homogeneous based upon SDS polyacrylamide electropherograms.

Using rhinovirus purified by isopycnic methods in metrizamide, Abraham and Colonno reported the differentiation and categorization of cellular receptors for 24 different Rhinovirus serotypes (34). The rhinoviruses were grown by infecting HeLa R-19 cells with an MOI of 1 PFU/cell in 75 cm^2 T-flasks. The infected cell cultures were incubated approximately 20 h at 34 C and harvested when the cells had detached from the flask surface. The cell suspension in growth media was freeze-thawed to release virus particles from the cells. The suspension was then clarified by centrifugation at 4,000 xg for 5 min. Polyethylene glycol 6,000 and NaCl were added to the supernatant at final

concentrations of 7 and 2.2% respectively. The mixture was stirred for up to 16 h at 4 C and the precipitated virus was recovered by centrifugation. The pellets were resuspended in a low ionic strength phosphate buffer. Sodium deoxycholate and nonidet P-40 were added at 0.3 and 0.6% respectively and the mixture was incubated at 4 C for 30 min. The suspension was clarified by centrifugation and the supernatant fraction containing the virus was layered over a 5 ml linear isopycnic density gradient of 40 to 60% (wt/vol) metrizamide in a buffer. Isopycnic banding of the virus was achieved by centrifugation for 24 h at 150,000 `xg. The viral bands, which were opaque, were harvested visually by aspiration. The fractions containing virus were diluted and pelleted by centrifugation. The pellets were suspended in small volumes of phosphate buffer and stored at -70 C. The isopycnically banded virus was reported as being essentially homogeneous and was used for studying binding to host cells. Three rhinovirus serotypes purified in this manner had infectivity to particle ratios (specific infectivity) more than 20 times higher than previous measurements determined with viruses purified in CsCl density gradients. The authors attributed this finding to the known binding of cesium chloride to viruses, a reaction that is known to alter the structural integrity of the virions and inactivate some viruses (38). It was stated by the authors that whether metrizamide or sucrose was used to purify the viruses, the same highly purified virus was obtained, however, the data for the preparations by sucrose gradients were not presented.

Mapoles et al. purified a HeLa cell receptor protein for group B Coxsackieviruses (35). Viruses were grown in Buffalo green monkey (BGM) kidney cells using a MOI of 10. After inoculation, the cells were incubated for 1 h at room temperature, overlaid with medium, and reincubated for 7-9 h. The cells were removed from the glass bottles by scraping with a rubber policeman and collected by centrifugation. The cells were resuspended in a buffer and freeze-thawed three times to release virus. The cell-virus suspension was clarified by centrifugation at 10,000 xg for 10 min. Sodium dodecyl sulfate was added to a final concentration of 1% w/v and the virus was pelleted by centrifugation at 100,000 xg at 18 C for 4 h through a 30% cushion of sucrose. The virus pellets were suspended in 0.5 ml of a buffer, diluted to 5.0 ml with 32.5% w/v CsCl, and centrifuged to equilibrium for 18 h at 40,000 rpm in a Beckman SW 50.1 rotor at 4 C. The gradients were collected by bottom puncture of the centrifuge tube, peak fractions of infectivity were pooled, and dialyzed to remove the cesium chloride. All virus preparations were stored in a vapor phase of a liquid nitrogen refrigerator. Virus prepared in this way was used for receptor experiments, and the purification and identification of the cell receptor complex. The final concentration of virus reported in this article was approximately 100 micrograms/ml. Based on the volumes recovered and the virus titer, 7.7×10^{11} PFU/ml was the calculated recovery from the gradients. No virus yield data or the numbers of viruses per cell were reported by Crowell. The purity of the virus was high, exceeding 90%, based upon autoradiograms in which the viral capsid

peptides were seen as the most abundant components. Crowell reported that there was copurification of some cellular material. The effect of the cellular material on the results of their experiments was not discussed.

The synthesis and structure of the capsid polypeptides of the 6 group B coxsackieviruses were compared in a report by Chatterjee and Tuchowski (39). Each virus type had to be purified to near homogeneity. The 6 group B coxsackieviruses were grown in suspension cultures of Hela S-3 cells at 37 C. Hela cells were concentrated and infected with 5-20 plaque forming units per cell and incubated at 37 C for 45-60 min. Following adsorption the cells were pelleted by centrifugation, resuspended in growth medium and the incubation continued. After 20 hours of incubation, when 60-80% of the cells exhibited cytopathic effects, the cultures were harvested. Harvesting and purifying cells at 8 h post infection also produced mature virions, however, the authors found a 20 h incubation provided a higher yield. The infected cell suspensions were frozen and thawed three times and centrifuged at 2,000 xg for 10 min. Each cell pellet was resuspended in a small volume of a buffer and sonicated for 3, 15 second bursts. The cell debris was removed by centrifugation. The supernatants were pooled and the virus in this fraction was pelleted by centrifugation at 150,000 xg in a Spinco 60Ti rotor. The resulting pellets were resuspended in 4-6 ml of a neutral pH buffer containing 0.5% Nonidet P40 and incubated at 4 C for 16 h to affect the complete dissolution of the viral aggregates. The virus suspension was clarified by low speed centrifugation and layered

over an 8ml linear CsCl density gradients. The gradients were centrifuged at 38,000 rpm for 3 h at 8 C in a Spinco SW 41 rotor. The virus in these isopycnic gradients was collected from the bottom of the tube and assayed for radioactivity and infectivity. Peak fractions were pooled and dialyzed in a phosphate buffer, pH 7.0, for 16 h at 4 C. The dialyzed virus suspension was diluted to 10.5 ml with a buffer containing 0.5% Nonidet P40 and 4.505 grams of solid CsCl. This mixture which was at an initial density of 1.324 g/ml was centrifuged to equilibrium at 38,000 rpm at 8 C for 18 h. Fractions were collected and the radioactivity, absorbance 260 nm and 280 nm, and refractive index for each fraction were determined. The peak fractions, based on the previous measurements, were dialyzed as before and stored in the vapor phase of a liquid nitrogen freezer. The virus purified by this double isopycnic cesium chloride method was found to be adequately purified for the study of capsid polypeptides. This purification was reported to yield $1\text{-}3\text{x}10^{11}$ PFU/ml. The virus/cell yield could be calculated from the data and was approximately 308 viruses/cell, based on an average of $6.5\text{x}10^{8}$ cells infected and a yield of $2\text{x}10^{11}$ viruses. The purity of the virus was greater than 95% based upon published autoradiograms and densitomeric tracings.

The advantage of one virus purification technique over another depends on the yield of virus and the degree of purity. All four methods selected for discussion here yielded picornaviruses in amounts necessary for the completion of the experiments and in adequate purity. The methods presented are typical of many such purifications and

represent the best of the procedures available for picornavirus purification. They differ not in general strategy or procedure but in a number of details depending upon the type of cell, the culture medium, the type of picornavirus, the extent of cell lysis required for maximum yield, the use of metrizamide or cesium chloride in isopycnic gradients, the use of detergents in the dissolution of virus from cell debris, and other factors not the least of which is the adaptation of the laboratory strain of virus to the cell chosen as the host. With one picornavirus group, the Rhinoviruses, yields which were 20-fold greater than reported by other researchers, were obtained by changing the isopycnic gradient material from the typical cesium chloride to metrizamide (34). This and other reports suggest that improvements in techniques could yield virus preparations of both better purity and higher specific infectivity.

MATERIALS AND METHODS

Cells

Buffalo Green Monkey (BGM) cells were obtained from Whittaker MA Bioproducts. African Green Monkey (VERO) cells were obtained as a seed culture from the American Type culture collection (ATCC CCL 81). Cells were grown in Dulbecco's modified Eagle's medium (DMEM)(Sigma) with 5% newborn calf serum (NBCS)(Sigma). The cells were grown in a 5% CO_2 humidified incubator at 37 degrees Celsius. Both cell lines were continuously propagated during these studies for 50-60 passages.

Viruses

Coxsackie B3 (CB3) Nancy was obtained from the American Type Culture Collection (ATCC VR-30). In order to produce large amounts of CB3, virus propagation was performed by infecting confluent BGM cultures in glass roller bottles. The bottles contained approximately $1.5X10^8$ BGM cells and were infected with CB3 at a multiplicity of infection (MOI) of 0.01-0.1. Virus was harvested 30 to 48 hours post infection when the cell sheets and individual cells became pycnotic or had lysed.

Radiolabeled CB3

Radioactive 35-S methionine (NEG-009H, New England Nuclear) was used to label CB3. Two confluent T-150 flasks of BGM cells were washed

twice with serum and methionine free DMEM and then incubated at 37 C for 1 hr in serum and methionine free DMEM. The media was decanted and 7.0 ml of DMEM serum and methionine-free media containing 0.01% NBCS and "35-S" methionine, (L-[35-S]-Methionine 37.0 MBq, 10-15 microcurries/ml) was added to each flask. CB3 was then added at a MOI of 0.02 and incubation was continued until all the cells detached, became pycnotic, or had lysed. The lysate was frozen and thawed three times and clarified by centrifugation at 5,000 xg for 15 min at 4 C. The supernatant containing radioactive virus was concentrated by $(NH4)_2SO_4$ precipitation. Room temperature saturated $(NH_4)_2SO_4$ in 0.01M Tris-HCL, pH 7.4 was added to the CB3 lysate on a volume for volume basis. The mixture was incubated on ice for two hours and the insoluble material collected by centrifugation (10,000 xg, 30 min, 4 C). The precipitate was dissolved in 1.4 ml of DMEM (1/10 the original volume) and dialyzed against 200 volumes of DMEM. The dialysate was adjusted to 1% NBCS and stored, if necessary, at -20 C. The concentrated virus preparation was purified by rate zonal centrifugation through 10 to 30% linear sucrose gradients in 0.01 M Tris-HCl pH 7.4. The fractions containing peak amounts of infectivity were pooled and dialyzed against 100 volumes of DMEM. The specific activity of the virus prepared this way was 100-300 pfu/cpm and the virus was greater than 90% pure based on SDS-polyacrylamide gel electrophoresis.

Standard Plaque Assay

The plaque assay for CB3 was essentially that described by Lewis et al. (40). BGM cells were grown in 55 cm^2 tissue culture petri dishes until they were 90 to 100% confluent. When the cells were ready, 0.1 ml from each tube of a 10-fold CB3 virus dilution (series 10^{-1} to 10^{-7}) was added, in duplicate to 2.9 ml of DMEM in the culture dish. The virus was allowed to adsorb to the cells for 1.5 h in a CO_2 chamber with constant rocking. When the incubation period was complete the inoculum was removed and the infected cells were overlaid with methylcellulose DMEM media (1.25% methylcellulose-4000, cP[0.1 P=0.1 Pa-s]). The overlaid plates were incubated for 24-36 h after which time the plaques were counted, without staining, using a X-Y transport carrier on a Nikon inverted phase contrast microscope.

The linearity of the plaque assay was determined from a plot of plaques versus virus dilution obtained from an assay linearity experiment. A stock of CB3 (1.0 $X10^7$ pfu/ml) was diluted in DMEM such that 0.1 ml of inoculum contained approximately 1000, 500, 250, 125, 67, 33, 16, 8, 4, and 2 pfu (2-fold dilution series). These dilutions were then inoculated, in duplicate, onto in the standard plaque assay. The plaques were counted the graph plotted with a best fit line through the data.

Adsorption Efficiency

The efficiency of adsorption of CB3 to BGM cells was obtained by a progressive adsorption experiment. A stock of CB3 was diluted in DMEM

so that 3.0 ml quantities of DMEM contained 1500, 1000, 500, 250, or 100 pfu. Each whole dilution was used as an inoculum on confluent monolayers of BGM cells in 100 mm^2 plates. The plates were incubated as in the standard plaque assay. At the end of the first adsorption period, each inoculum was totally transferred to a second set of five BGM plates and incubated as before. Following the second adsorption period, each inoculum was again removed and transferred to a third set of five BGM plates. Following incubation each inoculum was transferred to a fourth and final set of plates. All of the plates were overlaid and incubated 35 to 40 h, after which time the plaques were counted.

Statistical Consistency of Plaque Assay

The statistical consistency of the plaque assay was obtained by a replicative plating experiment. A stock of CB3 was assayed as per the standard assay only that five replicates were used at each dilution. The plaque numbers were used to obtain means, standard deviations, and a coefficient of variation.

Virus Purification

Several procedures were evaluated to optimize the purification of large quantities of CB3 from BGM cells. These procedures included several designed to study release and dissociation of CB3 from infected cell debris, concentration of virus, and rate zonal and isopycnic centrifugation.

CB3 Release From Cells

Freeze and thaw method. BGM cell lysates from 2 to 9, 850 cm^2 glass roller bottles (1.5 $X10^8$ cells each) were pooled and centrifuged (5,000 xg, 15 min, 4 C). The supernatant was decanted, saved and the pellet was resuspended in 1/10 the original volume of DMEM in one or more Corex glass centrifuge bottles. The pellets were dispursed by mixing and sonication (Branson Sonifier, 20,000 cps). The dispursed mixture was frozen at -20 C and thawed at 37 C. Some preparations were again frozen and thawed. Some preparations were frozen and thawed a third time. Following freezing and thawing the mixture was centrifuged at 5,000 xg for 15 min at 4 C. The supernatant was added to the original supernatant. The pellet was discarded. Virus plaque assays were conducted on the original lysate and the combined supernatants following freezing and thawing.

Salt extraction method. BGM cell lysates from one or two infected glass roller bottles, 40 or 80 ml, were collected and centrifuged as above. The supernatant fraction was decanted and the pellet mixed with 1/10 the original volume of 0.4 M NaCl in 0.01 M Tris-HCl, pH 7.4 until homogeneous. The mixture was sonified for 1min then centrifuged as before. The supernatant was combined with the first supernatant and the pellet extracted again with the high salt mixture. The resultant pellet was again extracted in the same manner. The combined supernatants were assayed for viral infectivity.

Sodium dodecyl sulfate extraction method. BGM cell lysates from 1 to 10 glass roller bottles were pooled. Sodium dodecyl sulfate plus 2-mercaptoethanol (SDS, 2-ME) was added to the lysate to a final concentration of 0.3 and 0.8% respectively. The mixture was incubated at 37 C for 10 to 30 min during which time the cellular debris solubilized. The mixture was chilled for 2 h at 0 C and the insoluble material and SDS were removed by centrifugation (10,000 xg, 20 min, 4 C). The supernatant which contained most of the virus was saved for assay and further purification.

Concentration of CB3 From Virus Lysates

The concentration of CB3 was accomplished by polyethylene glycol (PEG) precipitation, ammonium sulfate ($[NH_4]_2SO_4$) precipitation, or by pelletization by centrifugation.

PEG precipitation method. PEG precipitation was performed following the method of Abraham and Colonno (34). NaCl was added to the BGM lysates of CB3 to a final concentration of 2.2% (w/v). PEG-8000 (Sigma) was added to 7.0% (w/v). The mixture was stirred at 4 C on a magnetic stirrer overnight (12-16 h). The cloudy cell lysate was centrifuged (10,500 xg, 15 min, 4 C) and the supernatant was discarded. The resulting pellets were resuspended in "R" buffer (10 mM Tris-HCl [pH 7.5], 0.2 M NaCl, 50 mM $MgCl_2$, 10% [w/v] glycerol) by soaking for at least 3 h at 4 C . The suspension was further homogenized by trunulation and sonication. The suspension was assayed for infectivity by plaque assay.

Ammonium sulfate method. Ammonium sulfate precipitation was performed by adding equal volumes of room temperature saturated ammonium sulfate in 0.01 M Tris-HCl, pH 7.4, to the BGM cell lysates. The mixture was chilled on ice for 2 h and the insoluble material was collected by centrifugation (10,000 xg, 15 min, at 4 C). The resulting pellet was dissolved in 1/20 of the original volume of "R" buffer.

Centrifugation method. Pelletization of the virus by centrifugation was achieved by spinning the BGM lysates at 50,000 rpm in a Beckman L5-65 ultracentrifuge for 2 h at 10 C. The amber colored pellets were then resuspended in a minimal amount of "R" buffer by soaking overnight at 4 C in a refrigerator followed by trunulation and sonication. The pooled suspensions were assayed for viral infectivity and saved for further purification.

Isopycnic Centrifugation Purification Methods

All of the isopycnic procedures were performed in a Beckman L5-65 ultracentrifuge using a SW-41 swinging bucket rotor and Beckman polyallomer or polycarbonate tubes. Isopycnic centrifugation was performed with either of two types of centrifugation media. Metrizamide (2-[3-acetamido-5-N-methylacetamido-2,4,6-triiodobenzamido]-2-deoxy-D-glucose) (Nygarrd) was used in step gradients and preformed linear gradients, where as cesium chloride was used in self-forming gradients and preformed linear gradients.

Metrizamide step gradient procedure. Metrizamide step gradients were performed by filling 13.0 ml centrifuge tubes stepwise with 1.5 ml

60% metrizamide, 3.0 ml 20% metrizamide and up to 8.5 ml of clarified virus suspension, both dissolved in "R" buffer. The tubes were overlaid with paraffin oil as needed and spun for 3 h at 36,000 rpm at 10 C. Fractions were collected from the tube bottom by puncturing a hole and collecting the interfacial areas separately from the 60% and 20% material. The fractions were assayed for infectivity, density, and where appropriate radioactivity. Peak fractions were then pooled and stored.

Metrizamide linear gradient procedure. Gradient metrizamide runs were performed by pouring a 20-60% linear gradient (5 ml total) in "R" buffer. 5-8 ml of the clarified virus suspension was then carefully layered over the top of the gradient. The tubes were overlaid with paraffin oil as needed and spun at 36,000 rpm for 24-36 h at 10 C. Fractions were again collected by bottom puncture and assayed for infectivity, density, and when needed radioactivity. By either method the virus band could be seen by the presence of opalescent material in a definitive band with in the gradient media.

Cesium chloride linear gradient procedure. Cesium chloride gradients were prepared by dissolving cesium chloride to 944.8 mg/ml in "R" buffer with glycerol. An equal volume of the cesium chloride solution and clarified, concentrated virus suspension were mixed to yield a final cesium chloride concentration of 472.4 mg/ml with an average density of 1.34 g/ml. The resulting suspension was split between two centrifuge tubes, overlaid with parafin oil, and centrifuged

for 65-80 h at 31,000 rpm at 10 C. Gradients were collected by bottom puncture.

Preformed cesium chloride gradients. Preformed cesium chloride gradients were made by using definitive CsCl stock solution in "R" buffer. A 1.20g/ml solution was made by dissolving 2.8 grams CsCl in 10.0 ml "R" buffer. A 1.40 g/ml solution was prepared by dissolving 5.50 grams CsCl in 10.0 ml of "R" buffer. Solid CsCl was added to the clarified, concentrated virus suspension to produce a final density of 1.30 g/ml. At the time of centrifugation, gradients were prepared by successively layering 3.0 ml of 1.40 g/ml, 5.0 ml of 1.30 g/ml (virus suspension), and 2.0 ml of 1.20 g/ml CsCl solutions. The tubes were overlaid with parafin oil and centrifuged at 32,000 rpm for 55-70 h at 10 C. Cesium gradients were collected as before and assayed for infectivity, density, and radioactivity when necessary.

Both metrizamide and cesium chloride gradient peak fractions were pooled and subjected to infectivity assays, protein assays, and SDS-gel electrophoresis for purity determinations.

Rate Zonal Purification

Sucrose gradients were prepared to further purify cesium chloride banded virus. 10% and 30% sucrose in "R" buffer were used to pour 11.5 ml linear gradients in clear polycarbonate (Beckman) tubes for the SW-41 rotor. Up to 0.5 ml of virus suspension was layered on top of the gradients. The gradients were spun 3 h at 31,000 rpm at 15 C. The gradients were collected by bottom puncture. The tubes were assayed for

radioactivity, as necessary, and for infectivity. Peak tube fractions were pooled.

Protein Quantification

Protein concentration determinations were performed at each of the purification stages. The Quantigold procedure (Biorad) was used, using bovine serum albumin fraction V as a standard. The Quantigold reagent, 0.850 ml, was added to properly diluted fractions, standards, and controls in 0.150 ml. The mixture was vortexed at time zero. Following a 30 min incubation period, at room temperature for color development, the tubes were read at A^{595} using a Beckman spectrophotometer.

Radioactivity Measurement

Liquid scintillation was performed using a Packard liquid scintillation spectrometer. Liquid samples were dissolved in Aquasol II (NEN). Peak efficiencies were obtained with a gain of 6 and a window of 50 to 1000. Two sets of 10 min counts were averaged. In some experiments acid insoluble radioactivity needed to be determined. Trichloroacetic acid (TCA) insoluble material was collected on on a 0.2 micron cellulose acetate filters following a 15 min incubation of the material on ice. Each filter was washed twice with 10% TCA (5 ml). The filters were dried and placed into scintillation vials. Aquasol was added and the radioactivity was determined.

Polyacrylamide Gel Electrophoresis

The capsid proteins of CB3 were separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis according to the procedure described by Baum (41) with modifications by Johnson (42). Molecular weight markers and radiolabeled CB3 were placed in adjacent wells of a 10 or 12.5% acrylamide slab gels and run for 4 h or until the phenol red indicator moved to the end of the gel. The gels were stained overnight with Coomassie Blue and destained in a solution of 10% acetic acid and 40% methanol. Lanes containing radioactivity were cut into 1 mm sections placed in glass vials and hydrolyzed in 0.2 ml of 30% hydrogen peroxide (Sigma) overnight at 37 C. The solutions were mixed with aquasol and the radioactivity determined.

Autoradiograms were performed using Kodak Rpxomat film that were developed under the standard conditions.

The drying of polyacrylamide gels was performed using a Biorad model 543 gel dryer using the protocol outlined in the manual.

RESULTS

Assay

The consistency of the plaque assay for CB3 was studied. Using the protocol for the standard plaque assay, a stock of CB3 was serially diluted in DMEM+5% NBCS such that a 0.1 ml sample contained approximately 40 pfu. This dilution series and plaque assay was repeated five times to provide an estimate of the reproducibility of the plaque assay protocol. The consistency in the measurement of virus titer is presented in Table I. The extremes of the plaque counts were 28 to 56, with a mean of 42.1 and a standard deviation of 6.56. The enumerative plaque assay developed for CB3 and used in most of the experiments reported herein is thus capable of being performed with a high degree of consistency with variations around a calculated mean of approximately 15%.

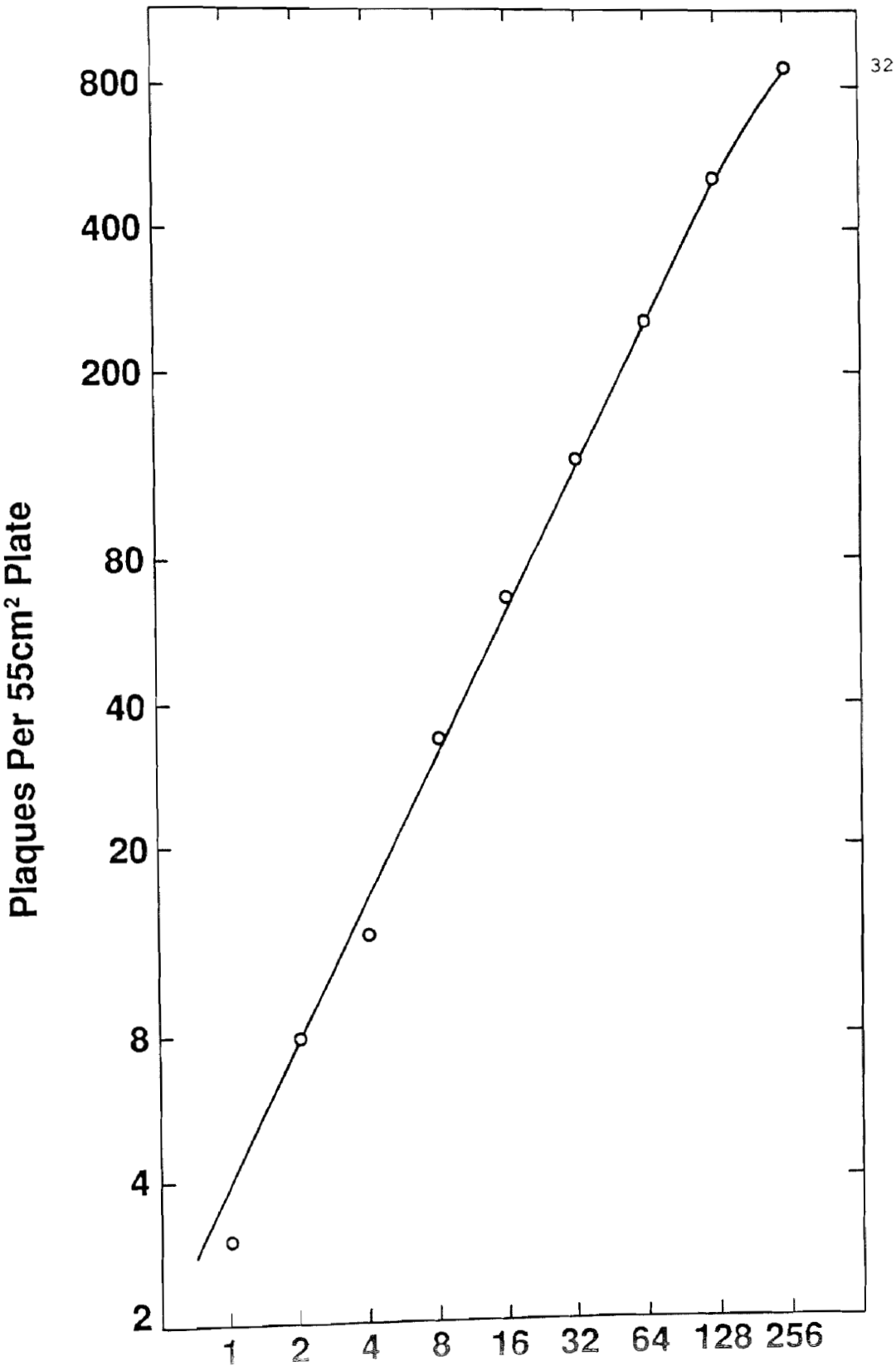
The plaque assay was demonstrated to have a linear response between 5 and 800 plaques on a 55 cm^2 plate when counted microscopically between 35 and 45 hours post-infection (Figure 1). Plaques consisted of 50 to 100 cells which were detached and round appearing relative to the normal, uninfected BGM cells. At this time of infection few cells had lysed. Approximately 12-24 h post-infection, perforations of the cell monolayer were easily observed. The linear response implies single-hit kinetics for this virus under under the conditions of the standard

Legend to Table 1. A stock of CB3 with an estimated titer of 1.1 X 10^8 pfu/ml was serially diluted with DMEM as follows; 1/100, 1/100, and 1/10. From the last dilution 0.05ml was added in triplicate to 2.95 ml of DMEM in 55 cm^2 tissue culture (TC) dishes for virus adsorption as in the standard assay protocol. The dilution series and virus plating was repeated five times. Plaques were counted microscopically using 40x magnification and from the dilutions and plaque count the titer was calculated for each plate. The area of the plate counted because of the templates used in the microscopic examination of tissue culture plates was 75.82% of the total area available.

Table 1. Consistency of Replicate Plaque Assays for CB3.

Dilution Set	Plaques on Each T.C. Disk (Plaques/Plates)	Calculated Titer of Virus Stock (pfu/ml)
1	41	1.1×10^8
	42	1.1×10^8
	44	1.2×10^8
2	43	1.1×10^8
	44	1.2×10^8
	46	1.2×10^8
3	28	0.7×10^8
	31	0.8×10^8
	40	1.1×10^8
4	42	1.1×10^8
	43	1.1×10^8
	44	1.2×10^8
5	39	1.0×10^8
	49	1.3×10^8
	56	1.5×10^8
$\bar{X}$	42	1.1×10^8
SD	6.5	1.7×10^7

Legend to Figure 1. Consistency of plaque assay. Twofold dilutions of a stock of CB3 were made using DMEM. Samples of each dilution were assayed in duplicate as in the standard assay protocol. Plaques were counted at 40 h post infection (PI). The dose response curve was prepared using the average of the plaque counts.



plaque assay. Single hit kinetics are observed for most viruses which do not require helper virus. Under these conditions, a single plaque forms due to the initial infection of a cell by a single, infectious virus particle followed by recruitment of adjacent cells by progeny viruses.

All of the studies performed with CB3 were done with a clonal isolate obtained from the original ATCC stock. Differences in the rate of plaque enlargement and plaque morphology were apparent. Plaques were observed to have diameters varying by a factor of 3 at 35 to 45 h postinfection. Also, it was common to observe variations in the density of infected cells in a plaque. The most common morphology was a complete and total involvement of all BGM cells within the plaque. Frequently plaques were observed which were diffuse, i.e., many normal appearing cells interspersed among definitively infected cells with the typical cytopathic effects. These variations could be attributed to the occurrence of CB3 variants with reduced or incomplete adsorption efficiency on BGM cells. Although other causes could not be eliminated, the efficiency of CB3 adsorption to BGM cells under conditions optimal for adsorption was measured.

The adsorption efficiency of CB3 is reported in Table 2. The mean efficiency of adsorption of CB3 in DMEM was 77.8% when the cell and virus mixture were incubated at an average pH of 7.4 in a humidified

Legend to Table 2. A stock of CB3 was diluted to contain 1000 pfu/0.75 ml DMEM+5% NBCS. All inoculations were performed in duplicate. The inoculum (0.75 ml) was added to a confluent plate of BGM cells which had been aspirated free of media. Adsorption was for 1.5 h at 37 C in a humidified CO_2 incubator. The plate was tilted at 15 min intervals to spread the inoculum.

Following the first adsorption period the plate was removed from the incubator and the inoculum was removed. Overlay media was added to the plate. The same inoculum used on the first plate was then added to another confluent plate for a second round of adsorption under the same conditions.

In like manner the adsorption was carried out using the same inoculum for one additional round of adsorption.

Following the addition of overlay medium the plates were incubated for 40 h and the plaques were counted as in Table 1.

Table 2. Adsorption Efficiency of CB3 on BGM Cells.

Adsorption Plate	CB3 Plaques Observed		
	Plate 1 (pfu/plate)	Plate 2 (pfu/plate)	$\bar{X}$
A - first adsorption	748	810	779
B - second adsorption	180	164	172
C - third adsorption	42	36	38
D - fourth adsorption	11	8	9

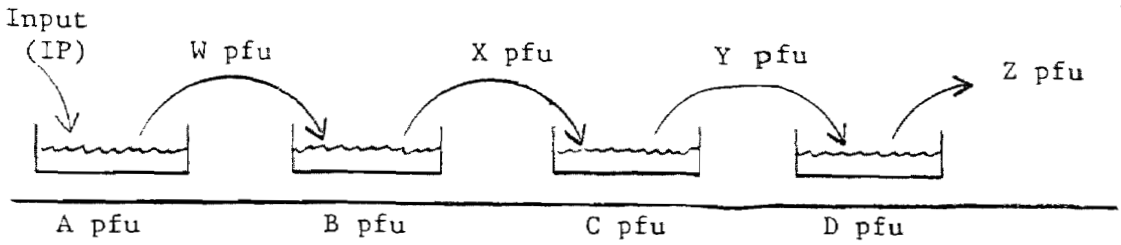
CO_2 incubator for 1.5 h at 37 C (Calculation set #1). Although the assay protocol used in this experiment (Legend to Table 2) was not identical to the standard plaque assay protocol, due to the nature of the experiment, a comparison of the two procedures using the same CB3 stock produced titers which were not significantly different from each other ($1.56x10^7$ pfu/ml versus $1.65x10^7$ pfu/ml).

The effect of a modification of the standard adsorption mixture was studied. An adsorption mixture consisting of phosphate buffered saline (PBS) containing 0.5 mM $MgCl_2$ and 0.9 mM $CaCl_2$ was substituted for DMEM+5% NBCS in the standard plaque assay. The titer calculated for the stock of virus assayed in PBS medium was 0.65 that obtained for DMEM+5% NBCS. Although other reports suggest that Ca^{++} and Mg^{++} are essential for maximal infection, apparently the DMEM+5% NBCS adsorption mixture was superior.

Virus Growth and Purification.

A primary consideration in virus yield and the purification of CB3 was to identify a method capable of releasing the maximum number of infectious viruses from intact, infected BGM cells. Table 3 presents the data obtained from single or multiple freezing and thawing of infected cultures following virus induced detachment of the cells from plastic or glass growth vessels.

Calculation Set 1. Calculations of Absorption Efficiency from Table 2.



Equations:

Y pfu = D pfu + Z pfu

X pfu = C pfu + Y pfu

W pfu = B pfu + X pfu

IP pfu = 1000 = A pfu + W pfu

Calculation:

Assume: 0.22 X 9 pfu (from D plates) = Z pfu

Then: Y = 9 + 2

X = 38 + 11

W = 172 + 49

IP = 1000 = 779 + 221

And Since Absorption Efficiency (A.E.) is Equal to

$$\frac{\text{\# plaques observed}}{\text{total input pfu}}$$

Then: A.E. for plates A = 779/1000 = 77.9%

A.E. for plates B = 172/221 = 77.8%

A.E. for plates C = 38/42 = 77.6%

and A.E. for plates D = 9/11 = 81.8%

Legend to Table 3. BGM cells at confluency in either T-flasks (TF), glass roller bottles (GRB), glass Blake bottles (GBB), or on glass beads in spinner flasks were infected with CB3 at an MOI of 0.001 to 0.02. Following detachment of the cells from the glass or plastic support, 30 to 40 h post infection (PI), the growth media containing the cells and debris was frozen and thawed at -20 C and 37 C, respectively.

The freezing and thawing cycle was repeated once or twice as indicated. The media was centrifuged at 10,000 xg for 15 min and the supernatant fraction was assayed as in the standard assay protocol. Cell numbers at confluency for the growth vessels were obtained by hemocytometer counts of duplicate, confluent, noninfected cell cultures ($1.37X10^7$ cells/TF; $2.33X10^8$ cells/RB, $7.0X10^7$ cells/GBB, and $1.2X10^8$ cells /spinner culture).

Table 3. Assessment of Freeze and Thaw Methods for the Release of CB3 from BGM Cells.

	Method	Culture[1] Vessel	Total Volume (ml)	Titer of Virus (pfu/ml)	Total Virus Amount (pfu)	Virus per Cell
1.	1 X FT	1 GRB	50	2.4×10^7	1.2×10^9	5.1
2.	1 X FT	1 GBB	40	4.0×10^6	1.6×10^8	2.3
3.	2 X FT	1 SC	150	3.0×10^5	4.5×10^7	0.4
4.	2 X FT	6 GRB	620	1.4×10^6	8.7×10^8	0.6
5.	2 X FT	7 GBB	212	4.6×10^6	9.8×10^8	1.9
6.	2 X FT	4 GRB	200	3.2×10^7	6.4×10^9	6.9
7.	2 X FT	10 GRB	450	2.5×10^7	1.1×10^{10}	4.8
8.	2 X FT	4 GRB	375	1.8×10^7	6.8×10^9	7.3
9.	2 X FT	5 GRB	224	6.5×10^6	1.5×10^9	1.3
10.	2 X FT	13 GRB	688	5.4×10^6	3.7×10^9	1.2
11.	2 X FT	4 GRB	192	5.4×10^6	1.0×10^9	1.1
12.	2 X FT	1 TF	6	3.3×10^7	1.9×10^8	13.8
13.	2 X FT	4 GRB	199	2.7×10^7	5.3×10^9	5.6
14.	3 X FT	4 GRB	192	4.9×10^6	9.4×10^8	1.0
15.	3 X FT	1 GRB	41	4.3×10^5	1.8×10^7	0.08
16.	3 X FT	2 GRB	99	1.2×10^7	1.2×10^9	2.6
17.	3 X FT	6 GRB	269	4.8×10^7	1.3×10^{10}	9.3

(1) GRB = Glass Roller Bottle ; SC = Spinner Flask
GBB = Glass Blake Bottle ; TF = Tissue Culture

The highest ratios of virus per cell were obtained for the two and three fold cycles of freezing and thawing. A maximum yield of 10-14 viruses per cell was obtained from cultivation of virus on BGM cells growing in either T-flasks or glass roller bottles. The overall average yield for the freeze and thaw procedures was 4.1 viruses per cell, with variation in yields of 20-fold on a per cell basis. There seemed to be no significant difference in yield between freezing and thawing two or three times.

Salt extraction of viruses from cells infected by CB3 did not produce yields which were significantly different from the freezing and thawing procedures (Table 4). Extraction of clarified lysate pellets with 0.4 M NaCl in R buffer yielded 24% more virus which had apparently been trapped or ionically bound with the material in the pellets (Table 5). A second extraction of once extracted clarified cell lysate pellets brought about an additional 5% of virus. There was, however, another 5% of assayable virus remaining in the pellet fraction. The total yield of virus prepared by salt extraction was only two times the average for the freezing and thawing procedures (8.6 viruses/cell).

The use of SDS in releasing of viruses from cells and cell debris resulted in yields of approximately 18 to 29 viruses/cell (Table 4). Little cellular debris remained microscopically visible following SDS treatment of the infected cultures, whereas, the other methods of virus

Legend to Table 4. BGM cells at confluency in T-flasks or glass roller bottles were infected as in Table 3. Following detachment, the cells were subjected to salt or SDS extraction as in Material and Methods. The media, clarified by centrifugation (10,000 xg for 15 min), was assayed for infectivity. The titers and yield of virus per cell were calculated based on a typical confluent yield of $1.37X10^7$ cells/T-flask and $2.33X10^8$ cells/glass roller bottle at confluency.

Table 4. Assessment of Salt and Sodium Dodecyl Sulfate Methods for the Release of CB3 from BGM Cells.

Method	Culture Vessel	Total Volume (ml)	Titer of Virus (pfu/ml)	Total Virus Amount (pfu)	Virus per Cell
3 X High Salt	1 GRB	95	2.1×10^7	2.0×10^9	8.6
SDS buffer	1 TF	7	5.3×10^7	3.7×10^8	27.0
SDS buffer	1 TF	10	4.0×10^7	4.0×10^8	29.0
SDS buffer	1 GRB	40	1.1×10^8	4.4×10^9	18.9
SDS buffer	1 GRB	39	1.4×10^8	5.5×10^9	23.7
SDS buffer	2 GRB	92	9.2×10^7	8.5×10^7	18.2

Legend to Table 5. BGM cells on two glass roller bottles were infected and the lysates frozen and thawed two times and clarified as in Materials and Methods. The pellet fraction was mixed with 0.4 M NaCl in R buffer (10 ml), sonicated, and centrifuged as in Table 4. The supernatant fraction was saved with the previous supernatant fraction and the pellet was again extracted as above. The resultant final pellet was resuspended in R buffer for plaque assay.

Table 5. Assessment of Repetitive High Salt Extraction of CB3 from BGM Cells.

Fraction	Volume (ml)	Titer of Virus (pfu/ml)	Total Virus Amount (pfu)	Percent of[1] Total Yield
Clarified Lysate	75	1.8×10^7	1.4×10^9	66.2
First 0.4M NaCl, R-Buffer Extract of Pellet	11	4.5×10^7	5.0×10^8	23.6
Second 0.4M NaCl, R-Buffer Extract of Pellet	9	1.2×10^7	1.1×10^8	5.1
Resuspended Pellet	3	3.9×10^7	1.1×10^8	5.1

(1) Total Virus Recovery - 2.1×10^9 pfu; pfu Recovered Per Cell Infected - 8.6

release resulted in much visible debris. It is likely that this debris either trapped virus or contained significant amounts of specifically bound virus.

Three methods were evaluated for the ability to yield a concentrated preparation of virus from the clarified cell lysates (Table 6). The precipitation of virus by polyethylene glycol (PEG) was found to be variably efficient in concentration of virus ranging from 11 to 98% of the virus in the cell lysate fraction used in the procedure. Both ammonium sulfate precipitation and centrifugation methods provided yields of approximately 85%, but the advantage of centrifugation is that it precluded the need for a dialysis step to remove PEG or ammonium sulfate prior to an isopycnic centrifugation step. Also, ammonium sulfate could not be used following the SDS procedure of releasing CB3 from infected cultures due to the formation of a large salt dodecyl sulfate precipitate. The yields of virus following centrifugation concentration varied over two-fold a range. This range likely reflects the extent of cell debris association of virus following several different extraction methods. Occasionally, more virus was recovered than originally calculated to be in the cell lysate (Table 6, items 8 and 9). This result was likely due to the breakup of viral aggregates or debris carrying multiple virus particles during the resuspension of the viral pellet.

Legend to Table 6. CB3 in cell lysates was concentrated by PEG preciptitaion, ammonium sulfate precipitation, and by centrifugation as described in Materials and Methods. The concentrated, resuspended virus was assayed for infectivity and the percent recovery was calculated.

. Assessment of Concentration Methods for CB3.

	Cell Lysate Reference Number (1)	Input Virus Volume (ml)	Total Virus Input (pfu)	Concentrated Virus Volume (ml)	Total Virus Recovery (pfu)	% Recovery
G Precipitation	3.	150	4.5×10^7	14.5	4.4×10^7	98
G Precipitation	4.	620	8.7×10^7	60.0	6.6×10^8	76
G Precipitation	8.	364	2.7×10^9	25.0	3.0×10^9	89
G Precipitation	7.	223	5.6×10^9	32.0	5.1×10^8	9
G Precipitation	5.	59	2.7×10^8	6.0	8.9×10^7	33
$(NH_4)_2SO_4$ recipitation	10.	71	4.6×10^8	0.3	5.5×10^8	119
$(NH_4)_2SO_4$ recipitation	12.	6	1.9×10^8	1.0	1.3×10^8	68
entrifugation	N.A.	450	1.1×10^9	51.5	1.5×10^9	136
entrifugation	N.A.	375	6.7×10^9	20.0	7.6×10^9	113
entrifugation	N.A.	44	6.1×10^9	6.0	5.8×10^9	95
entrifugation	17.	269	1.3×10^{10}	40.0	1.0×10^{10}	77
entrifugation	14.	190	5.1×10^9	17.0	3.6×10^9	71

h number refers to the cell lysate in Table 3.

The concentrated virus preparations were resolved by isopycnic centrifugation using either cesium chloride or metrizamide (Table 7). A consistent recovery of purified virus was not achieved by any of the methods employed. Cesium chloride methods (Table 7) resulted in recoveries between 4 and 35% and metrizamide procedures had infectivity recoveries between 4 and 24%. The higher recoveries were associated with a lower protein loading factor. Cesium chloride appeared to be capable of resolving the virus in higher protein concentrations than was metrizamide. The highest recoveries of 35 and 24% of the virus placed on the gradients for cesium chloride and metrizamide gradients, respectively, were associated with applied concentrations of protein of only 0.5 mg/ml of the resuspended cell lysate fraction. This data strongly suggested that a reduction of the total amount of protein applied to the gradients would produce a higher yield and possibly a more highly purified virus.

Recoveries of infectivity in isopycnic gradients could be improved by reducing the total amount of protein applied or by layering virus preparations which had been prepared by SDS lysis of infected cultures (Table 8).

A complete summary of three purifications of CB3 through cesium chloride is presented in Table 8. In procedure A, clarified cell lysate (CCL) was precipitated by ammonium sulfate. The CCL had been prepared

Legend to Table 7. CB3 cell lysates prepared by freezing and thawing were centrifuged at 10,000 xg for 15 min. The supernatant fractions were centrifuged to pellet the virus as in Materials and Methods. The resuspended virus was applied to either cesium chloride or metrizamide gradients as described in Materials and Methods. Virus assays were conducted on fractions collected from the gradients to find peak amount of virus and percent recoveries were calculated. Protein determinations were as described in Materials and Methods.

7. Assessment of Cesium Chloride and Metrizamide Isopycnic Gradient Procedures in the Purification and Recovery of CB3.

	Volume Applied (ml)	Total Protein (mg)	Total Virus Applied (pfu)	Volume Recovered (ml)	Total Protein Recovered (mg)	Total Virus Recovered (pfu)	Infectivity Recovered (%)
sium Chloride	10.0	10.1	3.8×10^9	2.6	0.91	5.1×10^8	13.4
sium Chloride	10.0	14.6	3.8×10^9	3.2	1.67	5.4×10^8	14.2
sium Chloride	19.5	10.1	5.1×10^8	5.5	2.75	1.8×10^8	35.2
sium Chloride	8.5	6.5	1.8×10^9	2.0	1.56	2.9×10^8	16.5
sium Chloride	9.7	17.9	9.5×10^9	4.6	2.8	4.2×10^8	4.4
trizamide Step	8.5	9.2	4.0×10^9	1.9	1.5	9.4×10^8	23.5
trizamide near Gradient	24.0	24.2	9.1×10^9	5.9	2.54	3.9×10^8	4.3
trizamide near Gradient	10.0	14.6	3.8×10^9	4.1	0.78	5.8×10^8	15.6
trizamide near Gradient	17.5	9.1	4.6×10^8	5.4	4.60	1.1×10^8	23.9
trizamide near Gradient	8.5	6.5	1.8×10^9	2.0	0.96	2.6×10^8	14.6

samples were layered over a 12 ml 10 to 30% linear sucrose gradient in R buffer. Centrifugation was at 30,000 rpm for 2 h at 15 C in the SW-41 rotor. Fractions, 0.5 ml, were collected by bottom puncture and assayed for infectivity. The peak tubes were pooled and assayed as above in A. The data obtained from the sucrose gradient was calculated to the total volume of CsCl pool and reported in the table.

C) 35-S labeled CB3 was prepared from BGM cells grown on one roller bottle. The conditions for labeling were as in B except that the media contained no serum. The virus was collected by pelletization and CsCl centrifugation as in A above.

Legend to Table 8. Summary of purifications of CB3.

A) CB3 purified as in Materials and Methods obtained from 35-S methionine labeled BGM cells on a single T-flask. CCL from SDS treated cells was mixed with an equal volume of saturated ammonium sulfate at 0 C and the precipitate collected after 1h at 0 C (10,000 xg for 15 min). The precipitate was dissolved in R buffer with glycerol (10%) and dialyzed against 100 volumes of R buffer with glycerol. The mixture was layered onto a discontinuous CsCl gradient (3.0 ml 1.40 g/ml CsCl in R buffer, 5.0 ml 1.30 g/ml CsCl in the dialyzed virus fraction, and 3.0 ml 1.20 g/ml CsCl in R buffer as the top component). The gradient was centrifuged 48 h at 20 C and 35,000 rpm in a Beckman SW-41 rotor. Fractions were collected from the bottom and assayed for infectivity. The fractions containing the maximum activity were pooled. The CCL, ammonium sulfate pool, and the CsCl pool were assayed for protein, infectivity, and radioactivity. The percent recovery and specific radioactivity were calculated.

B) 35-S labeled CB3 was prepared from BGM cells grown on two roller bottles. The cells were labeled using 100 microcurries of 35-S methionine in a medium containing 0.1% NBCS dialyzed against PBS. The cells were treated with SDS and the CCL prepared as in Materials and Methods. The pelletized virus was resuspended and layered as above onto discontinuous CsCl gradients which were centrifuged as in A. The peak fractions were pooled, dialyzed against R buffer w/o glycerol and 0.4 ml

Purification Yield Summaries.

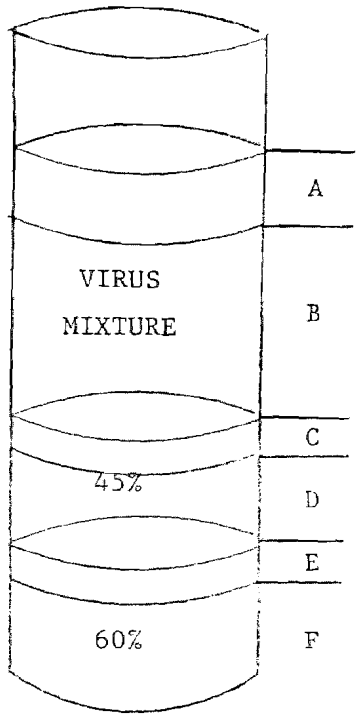
	Fraction Volume (ml)	Virus Titer (pfu)	Total Virus Amount (pfu)	Total Protein (mg)	Total TCA Label (cpm)	Infectious Virus Recovery (%)	Ratio Virus / CPM
	5.8	3.3×10^7	1.9×10^8	10.7	9.2×10^6	100	2.1×10^1
	3.8	3.2×10^7	1.2×10^8	8.2	6.3×10^6	63	1.9×10^1
ride ol	3.1	2.7×10^7	8.4×10^7	4.0	2.3×10^5	44	3.6×10^2
	4.4	1.5×10^8	6.6×10^9	8.1	3.4×10^8	100	1.9×10^1
	6.0	9.8×10^8	5.9×10^9	3.7	1.1×10^7	89	5.3×10^2
ride ol	4.6	6.1×10^8	2.8×10^9	1.1	6.7×10^5	43	4.2×10^3
ol	23.0	7.4×10^7	1.7×10^9	N.D.	1.3×10^5	26	1.3×10^4
	2.7	2.9×10^8	7.8×10^9	4.1	1.9×10^8	100	4.1×10^1
	2.0	3.6×10^9	7.2×10^9	2.0	3.1×10^7	92	2.3×10^2
ride ol	1.8	2.9×10^9	5.2×10^9	0.6	1.7×10^6	67	3.0×10^3

by SDS extraction of infected cells and cellular debris followed by dialysis to remove the SDS. Layering 8mg of protein in this fraction onto an 11ml cesium chloride gradient resulted in a 44% recovery of purified virus. In B, the cells were infected using DMEM containing 0.1% NBCS or a 50-fold reduction in serum proteins. Cells and debris were extracted with SDS in the usual manner and the virus was collected by centrifugation. The applications of 4.0 mg of protein to 11 ml cesium chloride gradients resulted in the recovery of 43% of the total infectivity. In C, no serum was used in the infection and the yield was approximately 67%. In these three procedures the overall average yield of infectivity through cesium chloride was 51%.

The polypeptide components of the various preparations were determined by SDS-gel electrophoresis. Estimates of the virus purity within these preparations could be obtained from structural studies of highly purified picornaviruses and, in particular, for CB3 it is known that this virus contains only four different structural polypeptides in equimolar amounts (37). Any other polypeptides observed in the electropherograms are considered impurities.

Discontinuous metrizamide gradients of the PEG precipitated CCL fraction produced the results in Figure 2. The largest amounts of virus, $1.2X10^8$ pfu, was found at the 45% metrizamide interface. Lesser amounts were in each of the other fractions. No fraction was found to

Legend to Figure 2. Discontinuous metrizamide gradients of pelletized and resuspended clarified cell lysates. A preparation of PEG, pelletized and resuspended (#3 of Table VI) CCL, was mixed with a preparation of 35-S methionine labeled cell lysate. There were a total of $3.5X10^8$ pfu and $2.6X10^7$ TCA precipitable counts per minute of label placed on the discontinuous metrizamide gradient (1.5 ml 60% metrizamide in R buffer as the bottom layer, 2.0 ml 45% metrizamide as the middle layer, and the remaining 7.5 ml was virus mixture diluted with R buffer). The total protein applied to the gradient was 14.6 mg. The gradient was spun for 3.5 h at 36,000 rpm at 4 C in a SW-41 rotor. Fractions were collected by aspiration with Pasteur pipettes. The total recovery of pfu was 78%, and the recovery of label 64%. The recovery of infectivity in the peak fraction was 34%.



Fraction	Fraction Volume (ml)	Total Virus (pfu)	Total Label (cpm)	Infectivity in Fraction (% of total) (1)
A	1.3	8.5×10^7	6.9×10^6	31
B	4.8	4.1×10^7	6.1×10^6	15
C	1.0	1.2×10^8	2.6×10^6	44
D	1.6	1.3×10^7	4.9×10^5	5
E	0.7	1.4×10^7	5.6×10^5	5
F	1.4	2.1×10^6	4.7×10^3	1

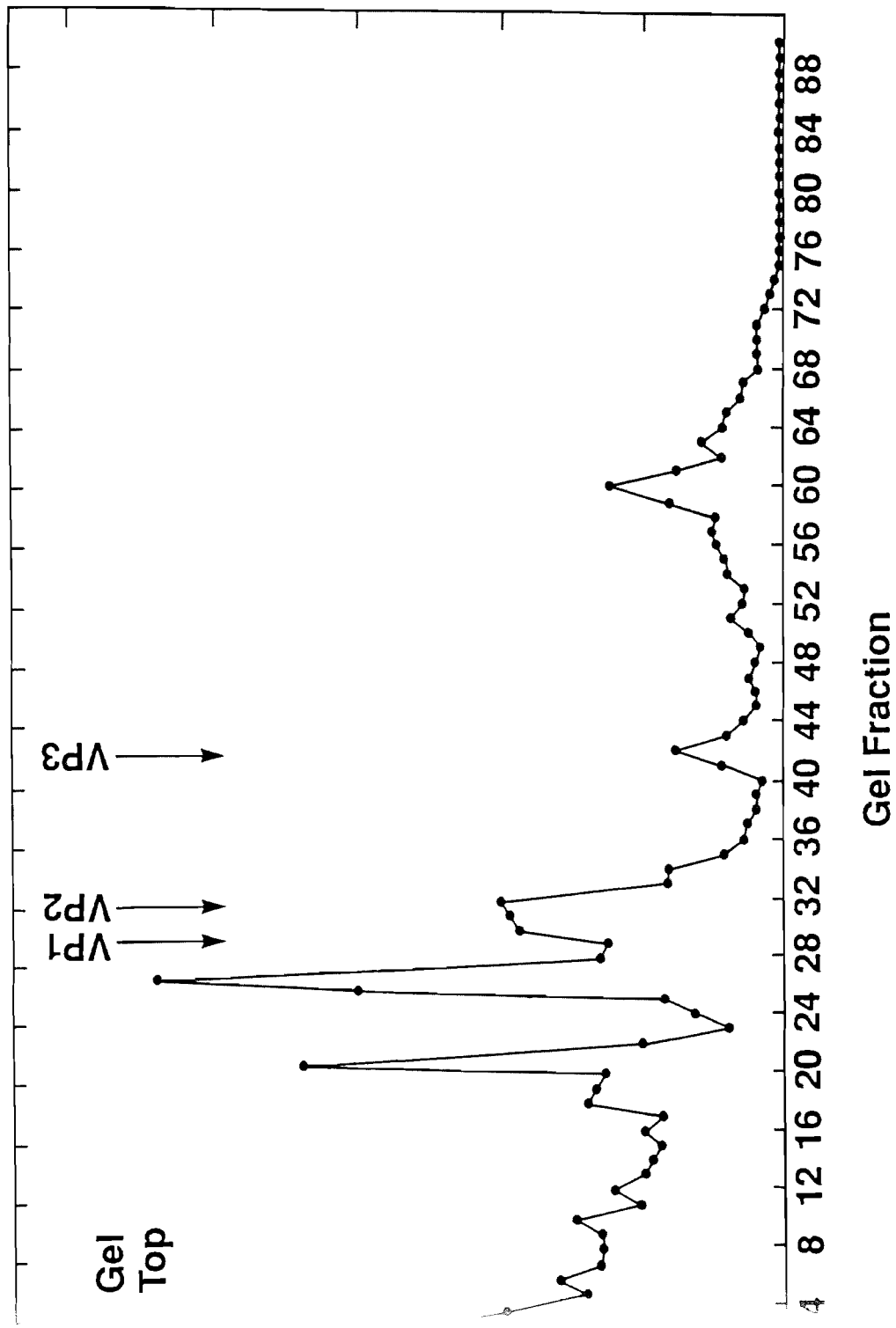
(1) The Total Infectivity Recovered (100%) was 2.7×10^8 pfu.

be free of virus as might be expected for a virus trapped in various amounts of cellular debris with a varying density. The yield of infectivity in the peak fraction, with respect to the total virus recovered, was 34%. This value is quite comparable to the 24% reported in Table 7.

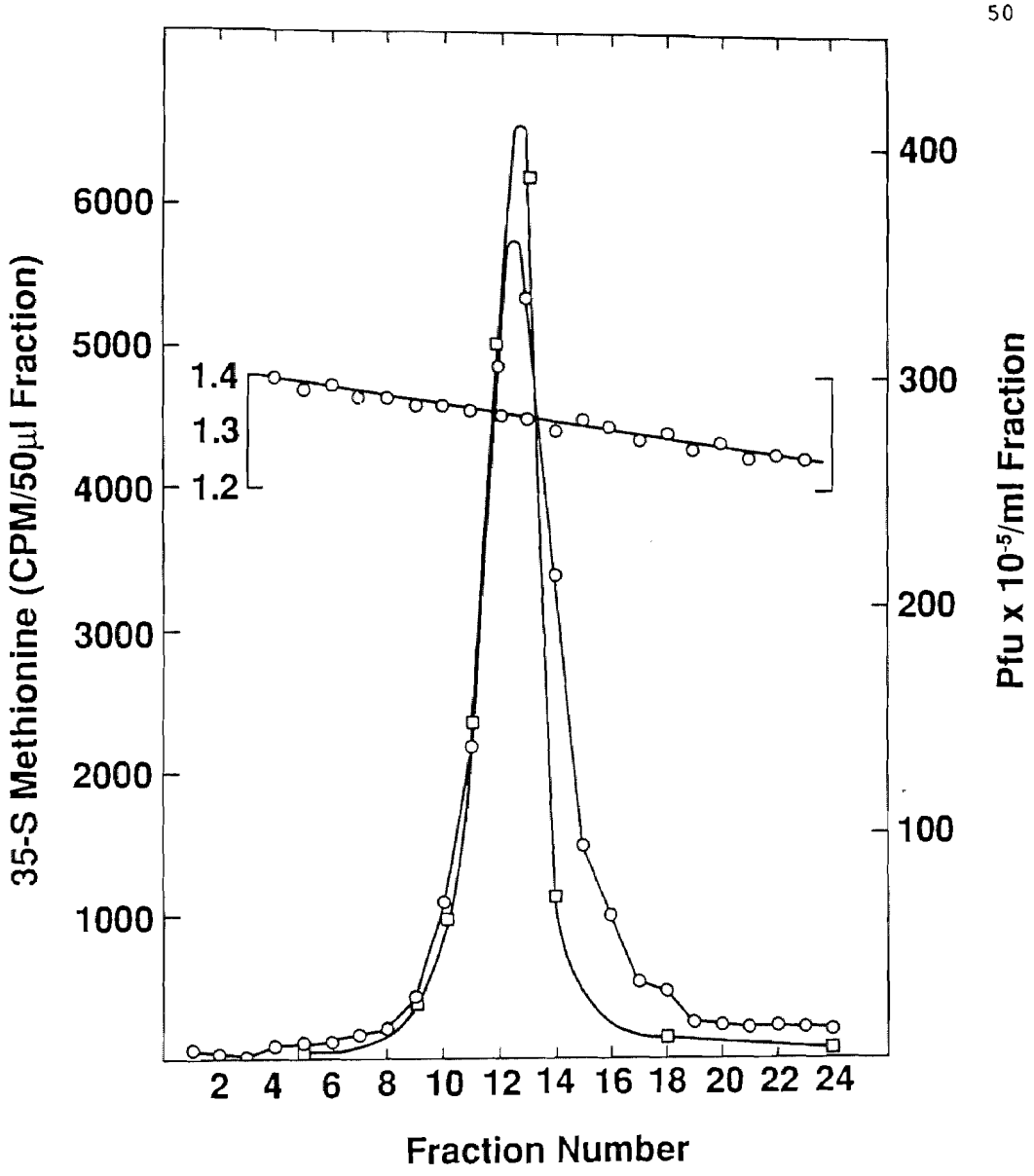
The peak fractions, however, were by no means pure. From a summation of radioactivity of fractions obtained from gel electrophoresis of the discontinuous metrizamide gradient fractions, Figure 3, the virus was maximally 10% pure. This calculation maximizes the purity of the virus due to the fact that only a small proportion of 10% of the label in the positions of VP1, VP2, and VP3 could represent viral polypeptides. Other cellular contaminants may have the same apparent molecular weight and migrate to the same position. The major contaminants appeared at 43 kd and 56 kd. There was also a large amount of contaminating protein that would not penetrate into the top of the gel.

Cesium chloride gradients of CB3 concentrated from CCL fractions produced both a 35-S methionine labeled protein pattern and a CB3 infectivity pattern which peaked at approximately 1.34 g/cc (Figure 4). The peaks of the methionine label and infectivity were not identical in shape and did not symmetrically overlap. This finding indicates some impurity in the gradient purified virus.

Legend to Figure 3. SDS-PAGE of C fraction from metrizamide gradient (Figure 2). Fraction C (50 microliters) was mixed with SDS sample buffer, heated and 25 microliters were applied to the 10% polyacrylamide gel and resolved as in Materials and Methods. The amount of label applied was $5.9X10^4$ cpm. The gel was sliced, dissolved and radioactivity determined as in Materials and Methods. The percent recovery of label was 57%. Marker molecular weight proteins in adjacent lanes were used to assign positions of VP1, VP2, and VP3.


Gel
Top
VP3
VP2
VP1
4 8 12 16 20 24 28 32 36 40 44 48 52 56 60 64 68 72 76 80 84 88
Gel Fraction

Legend to Figure 4. CsCl gradient centrifugation of pelletized virus resuspended in R buffer with glycerol. A sample (1.0 ml) of preparation 9 virus (Table 6) was mixed with pelletized and resuspended virus from a preparation labeled with 35-S methionine. Total label applied to the gradient was $1.8X10^5$ cpm. The total infectivity applied was $3.8X10^8$ pfu. The virus mixture was diluted to 5.0 ml in R buffer and solid CsCl crystals were added to yield an average density of 1.30 g/ml. The gradient was spun for 72 h at 35,000 rpm in the SW-41 rotor at 10 C. Fractions collected from the tube bottom were assayed for infectivity, radioactivity, and density. The recovery of radioactivity was 61% and the recovery of infectivity was 29%.

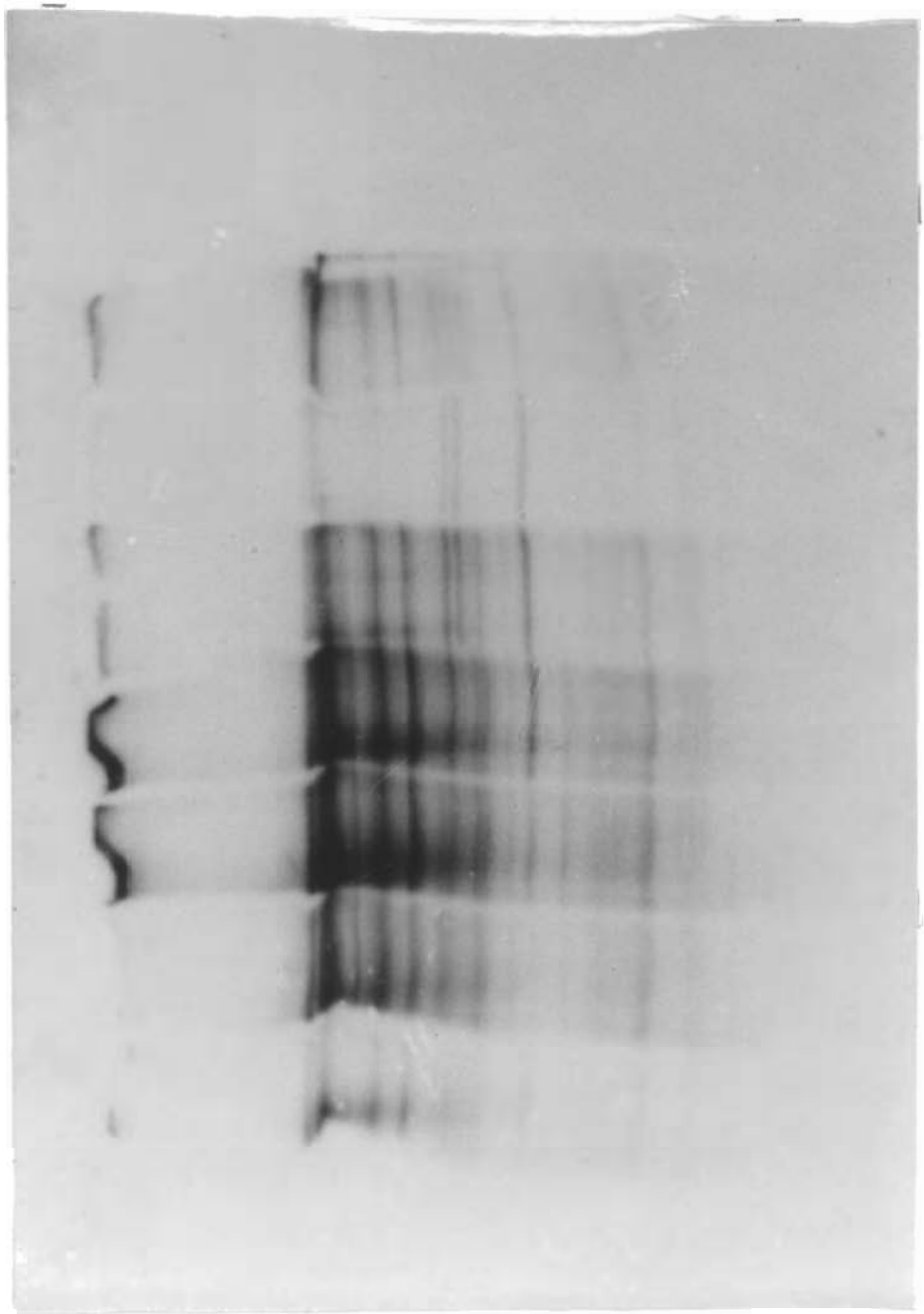

35-S Methionine (CPM/50µl Fraction)
6000
5000
4000
3000
2000
1000
1.4
1.3
1.2
400
300
200
100
Pfu x 10-5/ml Fraction
2 4 6 8 10 12 14 16 18 20 22 24
Fraction Number

SDS-gel electrophoresis of fractions across the peak of cesium chloride gradient purified virus revealed a large number of nonviral proteins (Figure 5). Only lane 6, corresponding to fraction 11 on the leading side of the infectivity peak, was sufficiently pure to clearly identify VP1, VP2, and VP3. VP4, the smallest of the CB3 structural polypeptides contains only 1-2 methionine residues (43) and cannot be observed on gel radioautograms without long exposures. The purity of the virus in lane 6 was estimated at 40% based on band density measurements.

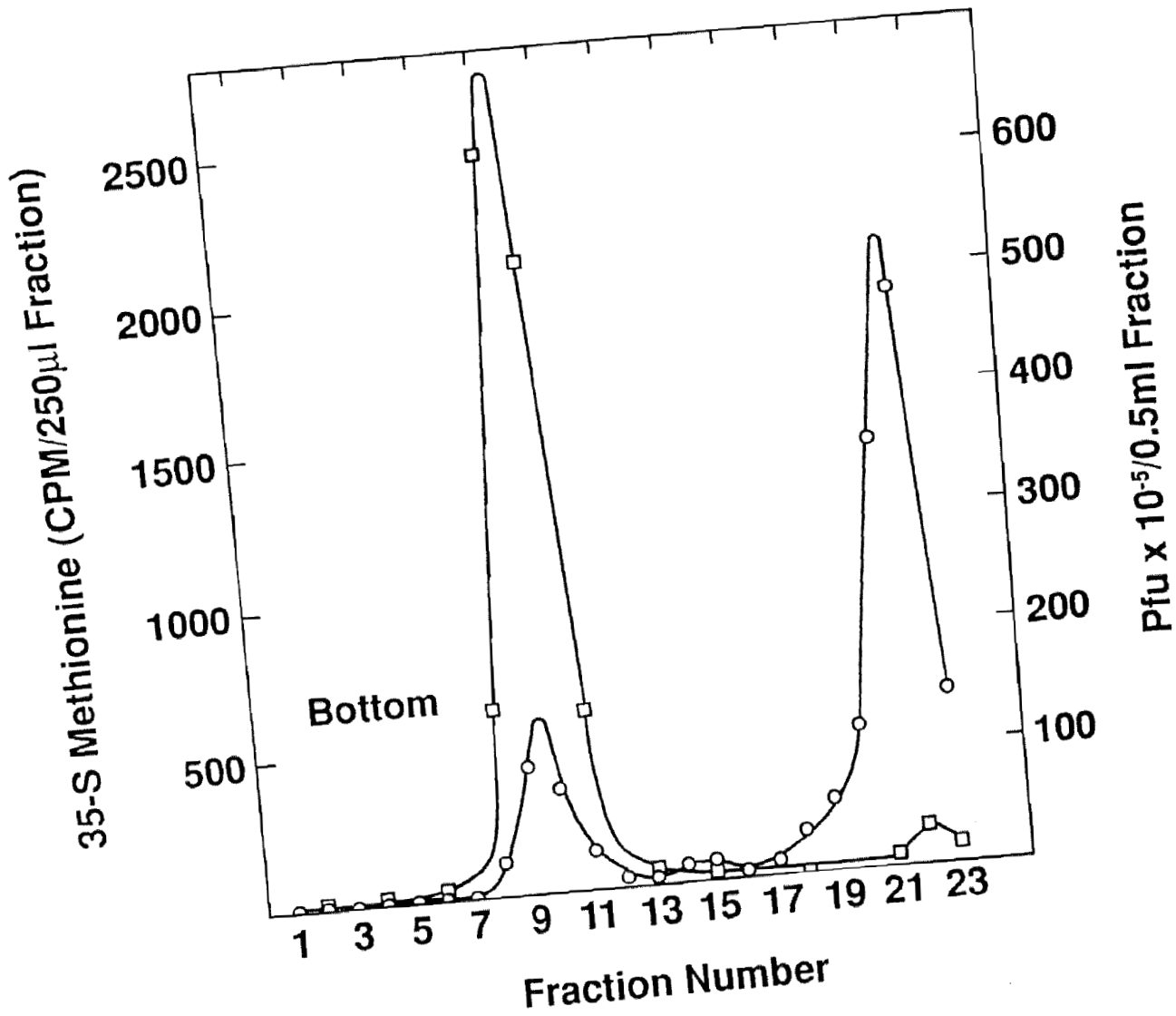
Very clearly another step in this purification process was necessary to obtain pure virus. Rate zonal centrifugation was selected as the next step in the purification process. Sucrose gradients were used to further purify cesium chloride gradient resolved CB3 (Figure 6). The sucrose gradient shown in Figure 6 reveals an identical, symmetrical, comigration of label and infectivity which is well resolved from other labeled protein. The peak fractions of infectivity were pooled (fractions 8-11) and were analysed by gel electrophoresis (Figure 7). VP1, VP2, VP3, and VP4 are clearly resolved. The purity estimated from the label in the bands relative to total label recovered on the gel was greater than 95%. This rate zonal and isopycnic gradient purified virus could be stored at -20 C for 2 months without loss in infectivity and was susbquently shown by others in this laboratory to be

Legend to Figure 5. SDS-PAGE radioautogram of a sample from the CsCl gradient (Figure 4). Sample (50 microliters) from fractions 11, 12, 13, 14, 16, and 18 and a pool of fractions 11, 12, 13, and 14 were mixed with 10X SDS-PAGE sample buffer (5 microliters) and resolved on a 12.5% SDS-gel as in Materials and Methods. The gel was dried and an autoradiogram prepared as described in Materials and Methods.

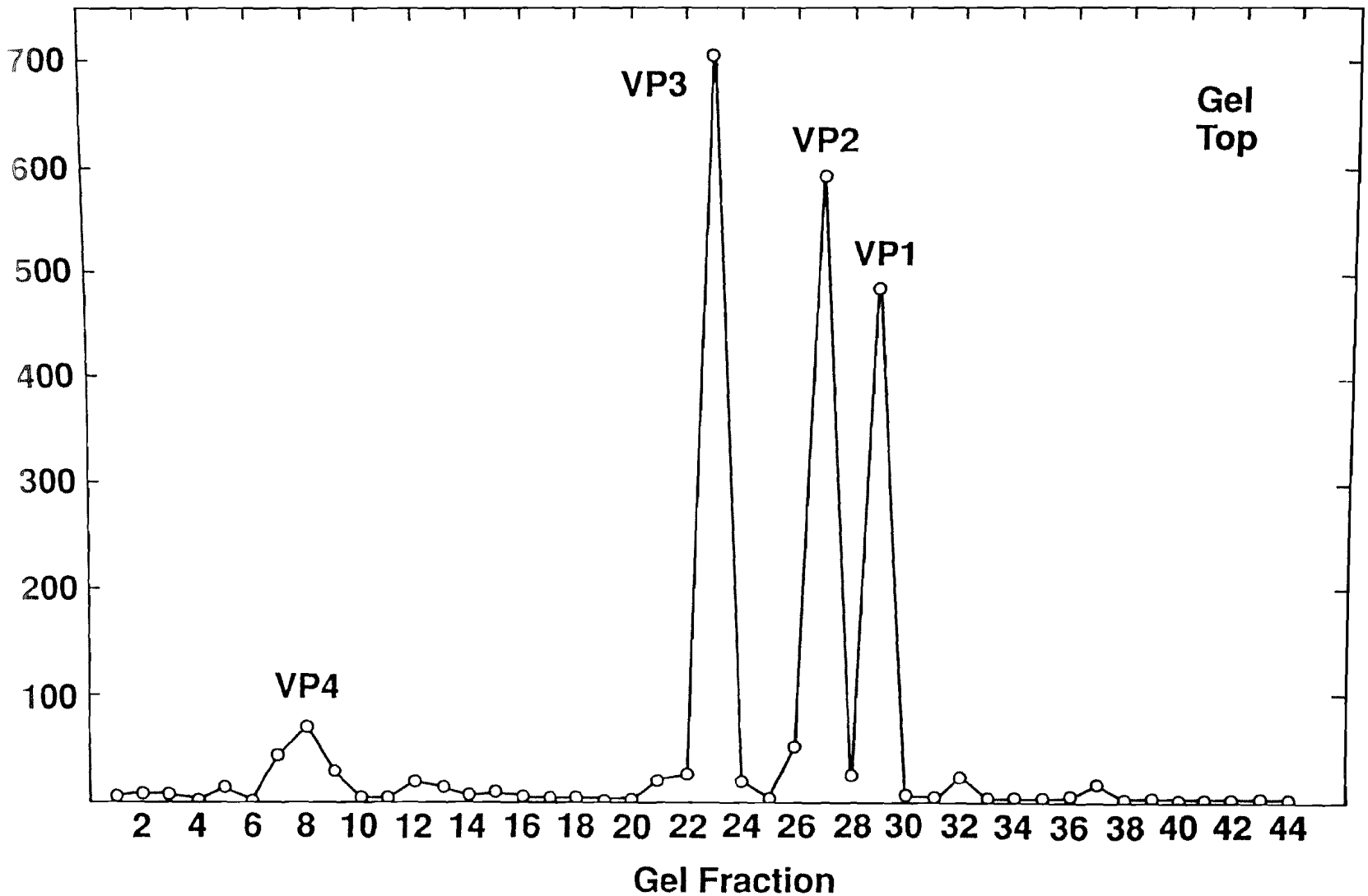
Lane	Fraction
1	18
2	16
3	14
4	13
5	12
6	11
7	pool of fractions as above



Legend to Figure 6. Sucrose gradient resolution of CsCl pooled CB3. Dialyzed, pooled CB3 (0.4 ml) from purification B (Table 8) was layered onto a 10 to 30% linear sucrose gradients prepared in R buffer as described in Table 8, B. Both radioactivity and infectivity were analyzed. Fractions 8-11 were pooled, dialyzed against R buffer and stored for PAGE analysis. The recovery of input label was 98%.


35-S Methionine (CPM/250µl Fraction)
2500
2000
1500
1000
500
Bottom
600
500
400
300
200
100
Pfu x 10-5/0.5ml Fraction
1 3 5 7 9 11 13 15 17 19 21 23
Fraction Number

Legend to Figure 7. SDS polyacrylamide gel electropherogram of pooled sucrose gradient fractions 8-11 (Figure 6). Pooled fractions (3,000 cpm, 6.0 ml from 3 sucrose gradients) were mixed with BSA (50 micrograms) and brought to a 10% trichloroacetic acid concentration. The mixture was incubated on ice for 1 h then centrifuged at 15,000 xg for 30 min at 0 C. The supernatant was discarded and the pellet rinsed once with ether and collected by centrifugation (15,000 xg, 30 min, 0 C). The pellet was dissolved in SDS sample buffer (50 microliters). The recovery of label was 80%. SDS-PAGE was performed on 12.5% slab gel as in Materials and Methods. The lane containing the labeled virus was cut into 2 mm sections and the radioactivity was determined as described in Materials and Methods. The final recovery was 71% of the input label.


700
600
500
400
300
200
100
VP3
VP2
VP1
VP4
Gel
Top
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44
Gel Fraction

useful in the study of virus adsorption to BGM cells (C. Teguh, personal communication).

DISCUSSION

The subject of this study has been the development of procedures and techniques for the purification of CB3 to homogeneity. Using techniques of differential, isopycnic, and rate zonal centrifugation CB3 has been purified sufficiently so that the virus was more than 95% homogeneous as measured by SDS-PAGE. The yield of virus through the purification has been greater than 40%. During the period of time to the completion of the purification process a number of parameters regarding the assay of CB3, its yield from various cell types and the interaction of CB3 with BGM cells were studied.

The plaque assay of CB3 was a modification of the procedure originally reported by Lewis et al. (40). CB3 was allowed to interact with cells at or near confluency for 90 min in standard DMEM growth medium containing 5% NBCS. The incubation was carried out in a CO_2 incubator at 37 C with plate rocking at 15 min intervals. The volume of the incubation mixture was approximately 0.1 the amount usually maintained in the plate or flask to increase the probability of virus and cell interactions. Although serum was not necessary for maximal infection, it was found not to interfere with the infection process. The major advantage of serum use was that the protein concentration reduced nonspecific bindings of CB3 with glass, micropipet plastics, petri dishes and tissue culture flasks themselves. Thus, the

reproducibility of the assay used in these studies was high. The variation in replicate assays was found to be only 15%.

Under the conditions of the standard assay developed in this thesis, linearity was achieved between 5 and 800 plaques on 55 cm^2 plates. The use of the inverted microscope facilitated the assay as plaques could be counted early when they were small. By waiting until the plaques became macroscopic, the linearity of the assay fell off beyond 80 plaques per 55 cm^2 plate. In any regard the linearity indicates single hit kinetics for the virus under the standard conditions of the assay.

The efficiency of virus adsorption to cells is usually measured using purified radiolabeled virus (34,37). In such studies, known amounts of stock virus are added to cells until saturation is achieved. Through these experiments it has been determined that CB3 adsorbs to Hela cells in a ratio of 100,000 particles per cell (31). This finding has been disputed microscopically (44). The difficulty is that such experiments contain mixtures of viruses, some capable of initiating infection, while most are incapable of causing infection. Under the best of purifications only 1 in 100 viruses within purified stocks is capable of causing infection.

In the adsorption experiment reported herein, the adsorption efficiency of infectious particles was established through the measurement of virus added to cultures and remaining in the same cultures following standard adsorption periods. This approach is an

improvement in that only virus particles capable of causing infection are measured. The adsorption of CB3 to BGM was not complete after 1.5 h of incubation. Approximately 78% of the virus was adsorbed. Neither reducing the period of adsorption to 1 h, or increasing it to 4 h changed the adsorption level of CB3 (Data not reported).

CB3 release from BGM cells was accomplished by one of three methods-freezing and thawing, high salt extraction, or SDS lysis. Freezing and thawing cells one, two, or three times released a maximum of 10 to 14 infectious viruses per BGM cell. The overall average for freezing and thawing one, two, or three times was 4.1 viruses per cell. Two cycles of freezing and thawing released more viruses than did a single cycle, however, there was no significant difference in yield between two and three cycles of freezing and thawing.

Multiple high salt extractions of BGM cells infected with CB3 produced yields which were not significantly different from freezing and thawing one, two, or three times. However, salt extractions of cell pellets resulting from centrifugation of clarified cell lysates resulted in the release of approximately 24% more infectious virus. This additional virus was potentially ionically bound to or mechanically trapped by cellular debris. Approximately 5% of assayable virus left in the pellet was not extracted. The total amount of infectious virus released per cell during multiple high salt extractions was 8.6 or about twice that for the average of the freezing and thawing methods.

SDS in R buffer with 2-mercaptoethanol was also used to release CB3 from BGM cells. The procedure released between 18 to 29 viruses per cell, a considerable improvement over the 4.1 virus per cell average achieved by freezing and thawing. Cell lysis by SDS left little cellular debris microscopically visible. Freezing and thawing and multiple high salt extractions both left large amounts cellular debris which could be sedimented by low speed centrifugation. It is probable that SDS reduced the amount of cellular debris capable of trapping CB3 and also disrupted ionic interactions binding the virus to particulate material.

Three methods were used and compared to concentrate CB3 from the clarified cell lysate. Use of polyethylene glycol precipitation as described by Abraham and Colonno (34) resulted in a broad range of infectivity recovery (11 to 98%). Ammonium sulfate precipitation of proteinaceous material from the clarified cell lysate produced recoveries of 68 and 119%. Virus pelletization by centrifugation resulted in an average recovery of 98.4% of the infectivity originally in the clarified cell lysates. Because of the simplicity and the relative speed with which centrifugation can be completed, centrifugation was chosen as one of the primary steps in the purification process.

The isopycnic gradient centrifugation methods used for resolving CB3 resulted in variable percent yields of virus infectivity recovered. Cesium chloride gradients were performed by preparing density steps of

cesium chloride in buffer with one density step (1.30 g/ml) containing a concentrated virus suspension. By using density steps of 1.40 g/ml, 1.30 g/ml, and 1.20 g/ml, equilibrium was achieved in the shortest amount of time. Cesium gradients performed in this manner yielded infectivity recoveries between 4 and 35% (Table 7).

Metrizamide gradients were done using either density step or poured linear gradients. Metrizamide step gradients were performed by layering 3.0 ml of 20% metrizamide onto 1.5 ml of 60% metrizamide in the bottom of a centrifuge tube. The concentrated virus suspension was then layered over the two layers of metrizamide. Linear metrizamide gradients were performed by pouring a 20-60% linear metrizamide gradient using a gradient forming device. A concentrated virus suspension was then carefully layered onto the top of the linear gradient. Metrizamide gradients performed by either of these two methods yielded infectivity recoveries between 4 and 24% (Table 7). The yields for both cesium and metrizamide procedures were disappointing.

The higher recoveries in both cases were most likely due to a lower amount of total protein in the gradient (Tables 7 and 8). Cesium chloride isopycnic centrifugation did appear to be able to resolve CB3 in higher protein concentrations than metrizamide (Table 7). In any regard, this data indicated that recoveries of virus could be improved by reducing the total amount of protein applied to the gradient.

Using the information gathered in the early studies and including the possibility of high protein concentration interference with

isopycnic gradient centrifugation three separate purifications were conducted through the cesium chloride step (Table 8). The first procedure released the virus from the infected cell monolayers by SDS lysis. The cells were grown and infected in media with the normal 5% bovine sera. CB3 was precipitated by adding equal volumes of room temperature saturated ammonium sulfate. The precipitated virus was collected by centrifugation, resuspended and dialyzed, and layered onto a discontinuous cesium chloride gradient. The total yield of infectivity was 44%.

The second procedure used CB3 which had been propagated in BGM cells using 0.1% NBCS or 1/50 the normal amount of serum proteins. The virus was released from the BGM cells by SDS treatment and concentrated by centrifugation. The concentrated virus was then layered onto discontinuous cesium gradients at a concentration which was not significantly different from the first procedure. The recovery was 43%.

In the last procedure, the virus was propagated without the use of serum in DMEM. CB3 was released by SDS treatment and concentrated by centrifugation as before. The concentrated virus suspension with a lower amount of protein was then layered on a discontinuous cesium chloride gradient. The recovery of infectivity for this procedure was 67%. Furthermore, by expanding the zone of recovery by several fractions on either side of the peak, most all of the infectivity could be recovered.

The virus preparations were analyzed by SDS gel electrophoresis to estimate the extent of virus homogeneity. The peak virus fractions from either cesium or metrizamide gradients were not sufficiently pure to be used in receptor-binding experiments. Many other polypeptide bands were present on the electropherograms besides those of the four known virus structural polypeptides. The purity varied greatly for the various preparations, however, cesium chloride isopycnic methods appeared to produce virus with the highest degree of purity.

In the study in which the serum was omitted from the infected cells, the peaks of ^{35}S-methionine and of infectivity are slightly off set (Fig.4). This finding suggests non-homogeneity, a finding which was confirmed by SDS-PAGE (Fig.5). Thus another step was added to the purification process. A rate, zonal centrifugation through a 10-30% linear sucrose gradient was performed subsequent to isopycnic centrifugation in cesium chloride. Analyses of these gradients demonstrated co-migration of ^{35}S-methionine and of virus infectivity. Furthermore, the virus was nearly (95%) homogeneous as measured by SDS-PAGE methods.

One question that arises is, why were so few infectious viruses produced by BGM and other cells? Other cell lines or laboratories have reported greater yields, for example, 335 CB3 particles per HeLa cell (39), 140 CB3 particles per YAC-1 mouse cell (45), and, more importantly, 100 CB3 particles per BGM cell (David P. Schnurr, personal communication). The highest ratio of virus produced per cell that this

lab obtained for CB3 on BGM cells was 29 using the SDS procedure. Several solutions to this question are possible. Although nonspecific nucleases and interferons are potential candidates for the inactivation of virus or the prevention of virus replication, respectively, it is unlikely that these would be differentially active in this CB3-BGM system and not in those listed above. It is possible, however, that the CB3 virions may have been ionically bound to the cell membranes, purified from the soluble fraction and, thus, rendered undetectable by plaque assay (46). It is also possible that many the virions have been packed together, i.e. not released from their iso-crystalline factories, resulting in aggregates which act during plaque assay as a single infectious particle (40). Both of these are quite probable because of the finding of increased release of virus by SDS.

High yields of virus from cell lines are invariably the result of selection of an adapted virus to a given cell line. The Nancy strain of CB3 has not been adapted to BGM cells. It, therefore, may be that CB3 cannot reproduce as readily in BGM cells as it does in HeLa cells (David P. Schnurr, personal communication). The isolate employed in this study was cloned in VERO cells following its usual propagation in HeLa cells. No attempt to select a clone highly productive in BGM cells was made before initiating these purifications.

A second question relates to how efficient this procedure was relative to procedures used by other investigators. Two common methods of comparison are degree of purity and percent of recovery. Relatively

few investigators have reported both numbers in any of the attempts at purification. Chatterjee and Tuchowski reported percent recoveries that ranged ranged from 67 to 87% (39). The degree of purity was not documented. No percent recoveries were reported by Crowell and Philipson, however, the virus purified by their procedures was greater than 90% based on SDS polyacrylamide electropherograms (37). Abraham and Colonno reported no numbers for percent recoveries for their purification procedure (34). However, they obtained infectivities more than 20 times greater than those received with cesium chloride by using metrizamide isopycnic centrifugation. The virus resulting from purification with metrizamide was apparently of very high purity.

The overall percent virus recovery for the procedures developed in this lab were approximately 40% through the zonal sucrose gradients. In the best of the cesium gradients, that were not overloaded, a yield of 67% was achieved. This recovery is not significantly different from that reported by Chatterjee and Tuchowski (39). It is to be expected that with higher virus per cell ratios that higher virus recoveries could be obtained. There were at times so little virus in several of my purification attempts that absorptive losses could account for half of the total virus available.

The importance of the group B coxsackieviruses relates to myocarditis. The six group B coxsackieviruses (CVB) have been shown to cause viral myocarditis (47). Infection of mice with CB3 has supported the etiology of CVB in human myocarditis (47). Other nonrelated

viruses, such as the echoviruses, influenza A and B viruses, and the Epstein-Barr virus, have also been shown to this syndrome in animal model systems (47). These viruses have been implicated in myocardial infections by elevated anti-coxsackievirus IgG and IgM antibody titers (47). These elevated antibody titers in conjunction with myocarditis caused by coxsackie B viruses strongly suggested that the group B coxsackieviruses are the etiologic agents of human myocarditis (47). Evidence directly relating CVB to human myocarditis by isolation of CVB from myocardial tissue is rare, however (47).

Cardiac cell destruction during CVB infection takes place by either direct cell infection and or by an autoimmune response. Cardiac cell death due to direct infection by CVB is due to the cytopathic effects of virus multiplication (48). The cells die due to virus mediated cell lysis (48). The heart tissue becomes necrotic with lesions that are subsequently infiltrated by mononuclear inflammatory cells (48). The main target of the immune system at this point appears to be the viral receptor on susceptible cardiac cells (48,49). These cells, once identified, are destroyed by cytotoxic T cells (49). The amplification of the response after CVB infection accounts for major cardiac tissue damage (48,49).

Another major factor influencing the extent of myocarditis is the genetic makeup of the infected individual (50). The genetic relationship is evident from studies of differing strains of mice which

develop differing degrees of myocardial infection when inoculated with CB3 (50).

No one mechanism appears to be solely responsible for cardiac cell destruction (49). However, it has been hypothesized that a combination of direct infection, autoimmune response, and the genetic make up of the individual, all affect the degree of myocarditis (49).

Because of the receptor's importance in infection by CB3 and its relationship to the immune targeting by toxic lymphocytes, there is a need to identify the virus cellular receptor. The identification of picornavirus cellular receptors have been brought about by biochemical purifications (VRa in HeLa cells infected by coxsackieviruses (35)) and by molecular biology approaches (ICAM-1 for rhinoviruses and PRm for polioviruses (31,33)). Cross-linking reagents of the type used for the study of membrane interactions with ligands such as hormones have not been successfully employed with viruses, but have been proposed as approaches to study the problem. The Denney-Jaffe reagent is an ^{125}I-labeled cross-linking reagent (N-[4(p-azido-m-[^{125}I]iodophenylazo)benzoyl]-3-aminopropyl-N'-oxysuccinimide ester) based on a cross-linking reagent developed by Denny and Blobel (51). The cross-linking reagent may be attached to antibodies or other ligands such as viruses. The complex is then exposed to the antigen or the cell. Once this is done the reagent is photoreactively cleaved and the labeled portion of the reagent becomes fixed to nearby membrane

components (51). The resulting cross-linked labeled cell components are then analyzed by gel electrophoresis and radioautography.

The cross-linking procedure has several advantages. The cross-linking process is easily controlled since the first step is chemical in nature and can be carried out in the dark independent of the second photoreactive cross-linking step (52). Cross-linking is very specific due to the covalent attachment of the reagents reactive group to the ligand which reacts specifically with cell receptors (52). Also, purification of the plasma membrane is not necessary, as with other methods to isolate the subcellular receptor, eliminating cross contamination with other subcellular fractions (52). All of the above procedures and methods require a purified supply of virus for the initial establishment of the technique. The process of purification has been the subject of this thesis.

In summary CB3 has been propagated in BGM cells and purified by differential, isopycnic, and rate zonal centrifugation to virtual homogeneity. The virus can be used in biochemical and molecular studies to deduce the cellular receptor.

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